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Vol
16
198

VOLUME 16

NUMBER 1

V. V. Frolkis, V. G. Shevchuk, S. F. Golovchenko, L. N. Bogatskaya, N. V. Verzhikovskaya and V. I. Medved	1	The effect of hormones on the haemodynamics in animals of different age groups
V. A. Dyundikova, Z. K. Silvon and T. L. Dubina	13	Biological age and its estimation. I. Studies of some physiological parameters in albino rats and their validity as biological age tests
M. Matsuo and K. Kaji	25	Aging of chick embryo fibroblasts *in vitro*—VI. Different aging patterns of population doubling rate and polyploidization
T. C. Kara and B. K. Patnaik	31	Age-related changes in endogenous respiration, respiratory control ratio and malonate inhibition of oxygen consumption of liver homogenate of male garden lizards
S. Hashimoto, T. Yokokura and M. Mutai	35	Effect of age and dietary composition on aortic collagen to elastin ratio and on lipid metabolism in F-344 rat
H. E. Enesco and D. Mahoney	41	Age related decrease in nuclear and nucleolar size in hypodermal cells of the rotifer
B. C. Wexler	47	Alloxan-induced diabetes in young vs old Sprague-Dawley rats
H. L. Gensler	59	The effect of hamster age on u.v.-induced unscheduled DNA synthesis in freshly isolated lung and kidney cells
S. S. Rath and B. K. Patnaik	69	Age-changes in basic protein contents of liver and muscle of the fresh water teleost, *Channa punctatus*
A. Turturro and S. A. Shafiq	81	A quantitative ultrastructural study of flight muscle in *Musca domestica* with experimentally lengthened lifespans
S. K. Kim, P. A. Weinhold, D. W. Calkins and V. W. Hartog	91	Comparative studies of the age-related changes in protein synthesis in the rat pancreas and parotid gland
J. Bochantin and L. L. Mays	101	Age-dependence of collagen tail fiber breaking strength in Sprague-Dawley and Fischer 344 rats

NUMBER 2

Editorial Announcement	107	George A. Sacher
H. V. Samis, B. J. Rubenstein, L. A. Zajac and S. M. Hargen	109	Temporal organization and aging in *Drosophila melanogaster*
I. Davies and A. P. Fotheringham	119	Lipofuscin—does it affect cellular performance?
R. G. Gosden, C. F. Holinka and C. E. Finch	127	The distribution of fetal mortality in ageing C57BL/6J mice: a statistical analysis
P. Rosen, A. D. Woodhead and K. H. Thompson	131	The relationship between the Gompertz constant and maximum potential lifespan; its relevance to theories of aging
T. H. Howell	137	Skin temperature of bedsore areas in the aged
P. L. Horn, J. T. Wood and J. J. Laver	141	Ageing changes in Porton rats with spontaneously arising pituitary tumors
S. Aizawa, N. Izumiyama and M. Kaiho	149	Role of cell surface morphology on age-related changes in concanavalin A-mediated red blood cell adsorption capacity of human diploid fibroblasts
R. S. Sohal and P. B. Buchan	157	Relationship between physical activity and life span in the adult housefly, *Musca domestica*
E. S. Szymanski, N. A. Little and D. Kritchevsky	163	Phospholipid metabolism in livers of young and old Fisher 344 and Sprague–Dawley rats

Vol. 16 1981

C. Viticchi and F. Szeszak	171	Change of nuclear protein phosphatase activity in the liver of old rats
Y. V. Pakin	177	On rational use of the cross-sectional method in analysis of the dynamics of biological indices with aging
K.-G. Collatz and S. Collatz	183	Age dependent ultrastructural changes in different organs of the mecopteran fly *Panorpa vulgaris*
J. L. Chertkov and O. A. Gurevitch	195	Age-related changes in hemopoietic microenvironment. Enhanced growth of hemopoietic stroma and weakened genetic resistance to hemopoietic cells in old mice
H. L. Gensler	199	Low level of u.v.-induced unsheduled DNA synthesis in postmitotic brain cells of hamsters: possible relevance to aging
Announcement	209	Satellite Symposium to ISN Meeting: Cellular and Molecular Mechanisms of Aging in the Nervous System

NUMBER 3

A. Comfort	211	Obituary—George A. Sacher
A. Ya. Litoshenko and E. L. Levitsky	213	Replication of total and mitochondrial DNA in rat liver with aging
V. K. Goyal	219	Early appearance and rate of lipofuscin pigment accumulation in human myocardium
P. B. Buchan and R. S. Sohal	223	Effect of temperature and different sex ratios on physical activity and life span in the adult housefly, *Musca domestica*
I.Zs.-Nagy, K. Nagy, V.Zs.-Nagy, A. Kalmár and É. Nagy	229	Alterations in total content and solubility characteristics of proteins in rat brain and liver during ageing and centrophenoxine treatment
K. C. Brahma and B. K. Patnaik	241	Effect of varied diet, restricted feeding and starvation on the characteristics of collagen of young garden lizards
D. D. Wheeler	247	Aging of membrane transport mechanisms in the central nervous system—2-deoxy-D-glucose transport in cortical synaptosomes from the Long–Evans rat
L. E. Rikans and B. A. Notley	253	Decline in hepatic microsomal monooxygenase components in middle-aged Fischer 344 rats
J. M. Capasso, T. M. Lemma and J. A. Zimmerman	261	Post-anoxic recovery of myocardial performance in senescent mice
K. Yamamoto, H. Kondo and M. Ohashi	271	A practical preservation method for human diploid fibroblasts in aging research
J. E. Fleming, H. A. Leon and J. Miquel	287	Effects of ethidium bromide on development and aging of *Drosophila:* implications for the free radical theory of aging

NUMBER 4

B. K. Patnaik and H. N. Behera	295	Age-determination in the tropical agamid garden lizard, *Calotes versicolor* (Daudin), based on bone histology
I. Szabolcs, G. Szilágyi, M. Góth, Zs. Kovács, M. Weber and T. Halász	309	Plasma triiodothyronine response to thyrotropin releasing hormone, thyrotropin and propranolol in old age
I. J. Singh and E. A. Tonna	317	Estimation of the turnover rates of ^{3}H-uridine in mouse skeletal tissues during aging
G. Schlager	325	Longevity in spontaneously hypertensive mice
M. K. Thakur and M. S. Kanungo	331	Methylation of chromosomal proteins and DNA of rat brain and its modulation by estradiol and calcium during aging
H. R. Massie, V. R. Aiello and T. R. Williams	337	Cadmium: temperature-dependent increase with age in *Drosophila*
A. Ralima	343	Vascular degenerative changes in the uterine arteries of senescent female golden hamsters
R. S. Sohal	347	Relationship between metabolic rate, lipofuscin accumulation and lysosomal enzyme activity during aging in the adult housefly, *Musca domestica*
H. E. Enesco and P. Kruk	357	Dietary restriction reduces fluorescent age pigment accumulation in mice

Vol 16 198

NUMBER 5

A.I. Frankel and E.J. Mock	363	Time course of hormonal response to sexual behavior in aging male rats
K. Kubota, R. Kubota, S. Takeda and T. Matsuzawa	371	Effects of age and sex of host mice on growth and differentiation of teratocarcinoma OTT6060
S. Mohan and E. Radha	385	Age related changes in muscle connective tissue: Acid mucopolysaccharides and structural glycoprotein
C.F. Holinka and C.E. Finch	393	Efficiency of mating in C57BL/6J female mice as a function of age and previous parity
H. Meshkinpour, M. Smith and D. Hollander	399	Influence of aging on the surface area of the small intestine in the rat
R. Das and M.S. Kanungo	405	Activity of guanylate cyclase of various tissues and its *in vitro* modulation of effectors as a function of age in rats
A.N. Bozcuk	415	Genetics of longevity in *Drosophila*: V. The specific and hybridised effects of *rolled*, *sepia*, *ebony* and *eyeless* autosomal mutants
	429	Announcement
	429	Erratum

Vol. 16 1981

NUMBER 6

B.J Merry and A.M. Holehan	431	Serum profiles of LH, FSH, testosterone and 5α-DHT from 21 to 1000 days of age in *ad libitum* fed and dietary restricted rats
M. Osanai and S. Kikuta	445	Age-related changes in amino acid pool sizes in the adult silkworm, *Bombyx mori*
W.E. Trout and W.D. Kaplan	461	Mosaic mapping of foci associated with longevity in the neurological mutants Hk^1 and Sh^5 of *Drosophila melanogaster*
S.N. Banerjee and S.R. Dasgupta	475	Age-related alteration of thyroidal and mesenteric mast cells of male albino rat
B.D. Dash and B.K. Patnaik	481	Effect of age and cold shock on the SDH activity of brain mitochondria and carbohydrate contents of brain of male garden lizard
G.C. Webster and S.L. Webster	487	Aminoacylation of tRNA by cell-free preparations from aging *Drosophila melanogaster*
S.S. Hotta	495	The influence of age on the increased activity of 3-hydroxy-3-methylglutaryl-CoA reductase in regenerating livers of partially hepatectomized rats

AUTHOR INDEX

Aiello, V.R., 337
Aizawa, S., 149

Banerjee, S.N., 475
Behera, H.N., 295
Bochantin, J., 101
Bogatskaya, L.N., 1
Bozcuk, A.N., 415
Brahma, K.C., 241
Buchan, P.B., 157, 223

Calkins, D.W., 91
Capasso, J.M., 261
Chertkov, J.L., 195
Collatz, K.-G., 183
Collatz, S., 183
Comfort, A., 211

Das, R., 405
Dasgupta, S.R., 475
Dash, B.D., 481
Davies, I., 119
Dubina, T.L., 13
Dyundikova, V.A., 13

Enesco, H.E., 41, 357

Finch, C.E., 127, 393
Fleming, J.E., 287
Fotheringham, A.P., 119
Frankel, A.I., 363
Frolkis, V.V., 1

Gensler, H.L., 59, 199
Golovchenko, S.F., 1
Gosden, R.G., 127
Goth, M. 309
Goyal, V.K., 219
Gurevitch, O.A., 195

Halasz, T., 309
Hargen, S.M., 109
Hartog, V.W., 91
Hashimoto, S., 35
Holehan, A.M., 431
Holinka, C.F., 127, 393
Hollander, D., 399
Horn, P.L. 141
Hotta, S.S., 495
Howell, T.H., 137

Izumiyama, N. 149

Kaiho, M., 149
Kaji, K., 25
Kalmar, A., 229
Kanungo, M.S., 331, 405
Kaplan, W.D., 461
Kara, T.C., 31
Kikuta, S., 445
Kim, S.K., 91
Kondo, H., 271
Kovacs, Zs., 309
Kritchevsky, D., 163
Kruk, P., 357
Kubota, K., 371
Kubota, R., 371

Laver, J.J., 141
Lemma, T.M., 261
Leon, H.A., 287
Levitsky, E.L., 213
Litoshenko, A.Ya., 213
Little, N.A., 163

Mahoney, D., 41
Massie, H.R., 337
Matsuo, M., 25
Matsuzawa, T., 371
Mays, L.L., 101
Medved, V.I., 1
Merry, B.J., 431
Meshkinpour, H., 399
Miquel, J., 287
Mock, E.J., 363
Mohan, S., 385
Mutai, M., 35

Nagy, E., 229
Nagy, K., 229
Notley, B.A., 253

Ohashi, M., 271
Osanai, M., 445

Pakin, Y.V., 177
Patnaik, B.K., 31, 69, 241, 295, 481

Radha, E., 385
Rahima, A., 343
Rath, S.S., 69
Rikans, L.E., 253
Rosen, P., 131
Rubenstein, B.J., 109

Samis, H.V., 109
Schlager, G., 325
Shafiq, S.A., 81
Shevchuk, V.G., 1
Silvon, Z.K., 13
Singh, I.J., 317
Smith, M., 399
Sohal, R.S., 157, 223, 347
Szabolcs, I., 309
Szeszak, F., 171
Szilagyi, G., 309
Szymanski, E.S., 163

Takeda, S., 371
Thakur, M.K., 331
Thompson, K.H., 331
Tonna, E.A., 317
Trout, W.E., 461
Turturro, A., 81

Verzhikovskaya, N.V., 1
Viticchi, C., 171

Weber, M., 309
Webster, G.C., 487
Webster, S.L., 487
Weinhold, P.A., 91
Wexler, B.C., 47
Wheeler, D.D., 247
Williams, T.R., 337
Wood, J.T., 141
Woodhead, A.D., 131

Yamamoto, K., 271
Yokokura, T., 35

Zajac, L.A., 109
Zimmerman, J.A., 261
Zs.-Nagy, I., 229
Zs.-Nagy, V., 229

Vol
16
19

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THE EFFECT OF HORMONES ON THE HAEMODYNAMICS IN ANIMALS OF DIFFERENT AGE GROUPS

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(*Received* 24 *April* 1980)

Abstract—The investigation was undertaken to study to effect of varying doses of epinephrine, vasopressin, insulin, thyroxin, estradiol-dipropionate on the haemodynamics and contractility of the myocardium of rats in different age groups, as well as the effect of epinephrine and vasopressin on coronary blood circulation in dogs of various age groups. The age group peculiarities of reactions to the hormones were compared with changes in their content in the blood of animals of different age groups.

Upon administering small concentrations of the hormones in this study, more pronounced changes in haemodynamics and contractility of the myocardium were observed in senile animals. The administration of larger doses led to more pronounced changes in mature animals. During the process of aging, there is a decrease in the range of the reactions of the cardiovascular system to the action of hormones.

In case of long term (14 days) administration of thyroxin, it was found that in senile animals the contractility of the heart was lower than that in short term (4 days) administration. In long term administration of thyroxin, it was found that in mature animals the index for the myocardium's contracting ability was more pronounced than in short term administration.

Irregular changes in the concentrations of different hormones in the blood, changes in sensitivity and ability of the myocardium to respond to their action lead to changes in the hormonal regulation of the heart, and to the development of aging metabolic and functional changes.

Vol.
16
1981

INTRODUCTION

MYOCARDIAL fibers and the heart as a whole come under double regulatory control—nervous and hormonal.

Our data on the impairment of nervous influences on the heart as well as on the changes in reactions to the transmitters were reported in *Experimental Gerontology* (1977) and *Experimental Aging Research* (1979). These shifts in the regulation of the cardiovascular system are in large measure linked with the destruction of nerve endings and changes in the intensity of synthesis and degradation of the transmitters.

The purpose of this paper is to study the reactions of the cardiovascular system to the administration of various hormones (epinephrine, vasopressin, thyroxine, insulin, estradiol-dipropionate), the comparison being made with subsequent changes occurring in general haemodynamics, myocardial contractile capacity and electrocardiographic shifts.

METHODS

The study is based on experiments on anaesthetized (Urethan 1 g/100 g) rats and dogs in various age groups: mature rats 8–10 months old, dogs 2–3 years old, senile rats 26–28 months, dogs 15 yrs and older. The thermodilution method was used for measuring haemodynamic parameters (minute and stroke volume, cardiac index and output index of the left ventricule, total peripheral vascular resistance) (Fegler, 1954; Gurevich *et al.*, 1965). Systemic arterial pressure, measured in the femoral artery, and pressure in the left ventricle were

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recorded using electromanometers of the Nichon Kohden firm, Japan and the EMG-01 (Hungary). Differentiation of the intraventricular pressure curve was achieved with a differentiator (Moibenko and Golov, 1973). The maximum rate of the build-up of intraventricular pressure dP/dt_{max} was estimated on the basis of the indices for intraventricular pressure and the differential curve. The maximum rate of myocardial fibre contractions was calculated according to the formula of Grunkemeir *et al.* (1974). The contraction index was calculated according to the method of Veragut and Krayenbuhl (1965). The sphygmogram, phonocardiogram and electrocardiogram were registered simultaneously. Based on these values, the phase structure of the systole of the left ventricle was determined.

Perfusion of the coronary vessels was conducted in dogs with closed thorax and natural respiration (Khomazyuk *et al.*, 1965). Catheterization of the coronary vessels was performed with a thin-walled metallic catheter with an obturator on the tip, the internal diameter of which was 1.5–2.5 mm. The catheter was introduced under oscilloscopic control through the left carotid artery and ascending aorta to the ostium of the left or right coronary arteries. The catheter was then introduced into the coronary vessel until it totally filled the lumen, which was reflected in a drop in blood pressure to 5–15 mm Hg, and perfusion was started instantly at a pressure 20–30 mm higher than arterial.

The overall content of tyroxin (T_4) in the blood was determined with the aid of radioisotopic packs Thyopac-4 (Amersham, U.K.), while the absolute quantity of free T_4 was calculated on the basis of the data on the overall content of T_4. The content of triiodthyronine (T_3) was determined using radioisotopic packs of CEA-IRE-Sorin, France. The absolute T_3 content was estimated proceeding from the overall content of T_3.

The insulin activity of the blood and the activity of the free fraction of insulin in the blood were determined by the method based on insulin's ability to stimulate glucose absorption by the isolated diaphragm of the rat (Antoniades, 1958). Immunoreactive insulin was determined using the packs of CEA-IRE-Sorin, France, according to the accompanying instructions.

The concentration of vasopressin in the blood was measured by a biological method, according to the antidiuretic effect in alcohol anesthetized and constantly hydrated rats (Czaczkes *et al.*, 1964; Gusek and Lesnik, 1968). Diuresis was registered to an accuracy of 0.02 ml, employing a unique five-channel unit for determining vasopressin in the blood (Golovchenko *et al.*, 1977).

The content of epinephrine (E) and norepinephrine (NE) in the blood was measured by a modified version of the trioxyindole method (Matlina, 1961), based on the Vendsalu method of processing blood (1960), isolation and oxidation to the adrenochrome level by the Euler and Lishajko method (1961). Fluorescence intensity was measured using a highly sensitive fluorimeter FO-1. The obtained data were then processed by statistical methods with the use of the Student *t*-test criterion.

RESULTS

With increasing age, the epinephrine content in the blood of rats increases from 0.697 ± 0.023 μg/l. in mature rats to 1.035 ± 0.086 μg/l. in senile rats. On the other hand, there is a corresponding drop in norepinephrine content from 1.115 ± 0.011 to 0.154 ± 0.072 μg/l. Therefore, it may be said that the overall content of catecholamines in old age is somewhat lower.

The diversified shifts in the content of cathecholamines in the blood result in a sharp drop in the age coefficient $N\varepsilon/\varepsilon$: in one-month-old animals it is 5.88, in mature animals it drops to 1.59, and in senile rats it is only 0.15 ($p < 0.001$).

During the study of the effect of epinephrine on haemodynamics, the hormone was administered in a broad range of doses—from 0.001 to 10 μg/100 g of bodyweight. The experiments demonstrated that for the administration of 0.05 μg/100 g of epinephrine to rats, it was possible to observe in the senile specimens a statistically significant increase in the minute and stroke volume, arterial pressure, cardiac index and the output index of the left ventricle. The administration of a similar dose of this hormone to mature specimens did not result in any changes in the activity of the heart. Significant changes in them were observed only upon increasing the dose of epinephrine to 0.15 μg/100 g. The administration of the hormone in such a quantity leads to changes in the haemodynamic parameters (Table 1). From the table, it is evident that the administration of small doses of epinephrine leads to an increase in the minute volume, cardiac index and output index of the left ventricle; there is a rise in intraventricular pressure, a maximum rate of build-up of intraventricular pressure, a maximum rate of myocardial fiber contractions and the contraction index.

Vol. 16 1981

TABLE 1. INFLUENCE OF EPINEPHRINE ON HAEMODYNAMICS AND CONTRACTILE FUNCTION OF THE MYOCARDIUM IN RATS OF DIFFERENT AGE (MEAN VALUE ± STANDARD ERROR)

Indices	Adult: Control	Adult: epinephrine 0.05 mg/100 g	Adult: epinephrine 0.15 mg/100 g	Old: Control	Old: epinephrine 0.05 mg/100 g
Systemic arterial pressure (mm Hg)	79.7 ± 3.2	+5.9 ± 1.4*	+11.0 ± 1.7	84.2 ± 2.6	+13.9 ± 2.0
Cardiac output (ml/min)	92.6 ± 3.0	+9.8 ± 2.1*	+24.1 ± 5.0	76.2 ± 2.4	+21.9 ± 3.2
Heart rate (beats/min)	349 ± 21	+6.1 ± 3.8*	+12.6 ± 4.2*	335 ± 10	+15.8 ± 3.6*
Stroke volume (ml)	0.26 ± 0.02	+0.02 ± 0.001*	+0.06 ± 0.01	0.22 ± 0.02	+0.05 ± 0.01
Cardiac index (l/min/m^2)	1.890 ± 0.02	+0.204 ± 0.02	+0.620 ± 0.03	1.342 ± 0.06	+0.547 ± 0.02
Left ventricular minute work index (kg/m^2)	2.016 ± 0.18	+0.246 ± 0.03	+1.034 ± 0.04	1.533 ± 0.03	+0.909 ± 0.02
Systemic vascular resistance (dyn · s · cm^{-5})	70360 ± 8319	−2119 ± 859*	−8145 ± 1382*	90757 ± 10512	−8919 ± 1421*
Left ventricular pressure (mm Hg)	89.7 ± 3.1		+14.2 ± 2.1	92.0 ± 3.0	+16.8 ± 1.3
Maximal rate of rise of left ventricular pressure (mm Hg/s)	3378 ± 250		+946 ± 147	2650 ± 189	+953 ± 123
Contractility index (Relative Units)	60.0 ± 2.3		+8.7 ± 0.1	47.4 ± 3.9	+10.8 ± 0.2

Note: + increase; − decrease.
*Statistically nonsignificant, $p > 0.05$.

By boosting the doses of hormones administered, it was possible to observe an oppositive relationship in the haemodynamic changes. For instance, the administration of a dose of 5–10.0 μg/100 g of epinephrine resulted in a lowering of the minute volume, cardiac index and output index of the left ventricle. Arterial pressure and total peripheral resistance of the vessels increased, more substantially in mature specimens. Therefore, small doses of epinephrine in senile rats actually did lead to changes in heart activity. Upon administration of large doses, the changes were more pronounced in mature specimens. In experiments on dogs it was shown that in senility there is a change in the reaction of the coronary vessels to epinephrine. For example, dilatation of the coronary artery developed in senile dogs upon administration 0.007 ± 0.001 μg of epinephrine to the coronary bloodflow, whereas in mature specimens this effect was achieved by 0.01 ± 0.003 μg. As the concentration of epinephrine increased (0.1 μg), in mature specimens the drop in the tone of the coronary vessels continued to increase, whereas in senile dogs, a paradoxical reaction was observed—there was an increase in the tone of coronary vessels (Fig. 1). In this case, in senile dogs, unlike in mature specimens, instead of a rise, there was a drop in the contractile ability of the myocardium. For example, the maximum rate of build-up of intraventricular pressure fell by 527 ± 68 mm Hg ($p < 0.05$), the maximum rate of myocardial fiber contractions fell by 1.3 ± 0.4 of muscle length ($p < 0.05$). The contractile index fell by 3.2 ± 0.8 relative units ($p < 0.02$).

Vasopressin

With an increase in age, the vasopressin content in rat blood rises from 7.4 ± 2.0 μU/ml plasma in mature specimens to 28.0 ± 2.4 μU/ml in senile specimens.

The administration of vasopressin leads to pronounced haemdynamic changes (Table 1). From the table, it is evident that vasopressin in a dose of 0.02 U/100 g results in a marked drop in the minute volume, cardiac index, a growth in the total peripheral resistance of the vessels, and a more substantial growth in senile rats.

A study of the phase structure of heart activity upon administration of the hormone revealed that under the effect of vasopressin (0.02 U/100 g), there was a 51.2% longer period of tension in senile specimens, while in mature specimens the corresponding figure was 23.8%. This is due both to the longer asynchronous contraction phase (in senile specimens by 26.5%, in mature specimens 21.3%), as well as to isometric contraction which changes more significantly in senile specimens (by 146.9%) than in mature (by 51.9%). The myocardial tension index increases in senile specimens (by 16.5%) and undergoes no changes in mature specimens under the effect of vasopressin. Upon administration of the hormone, the period of expulsion is prolonged both in senile and mature specimens. There is a tendency for the intrasystolic expulsion index to drop.

The administration of vasopressin leads to objectively ECG recorded manifestations of coronary insufficiency. Signs of myocardial hypoxia (increased T wave, lowering of the S-T segment) appear in senile specimens upon administration of smaller doses of vasopressin. For example, when a dose of 0.005 U/100 g of hormone was administered, there were no changes in the ECGs of mature specimens, whereas in senile specimens there was a change in the size of the T wave (in 60% of the cases), and a shift in the S–T segment (in 40% of the cases). A somewhat larger dose of vasopressin (0.02 U/100 g) was found to induce a higher T wave by 172.5 ± 21.5 mV, whereas in mature specimens the rise was only 97.5 ± 6.5 mV; the shift of the S–T segment was more significant in senile specimens (by 160.0 ± 15.6 mV) than in mature specimens (by 80.2 ± 10.0), which testifies to more pronounced myocardial hypoxia in senile rats.

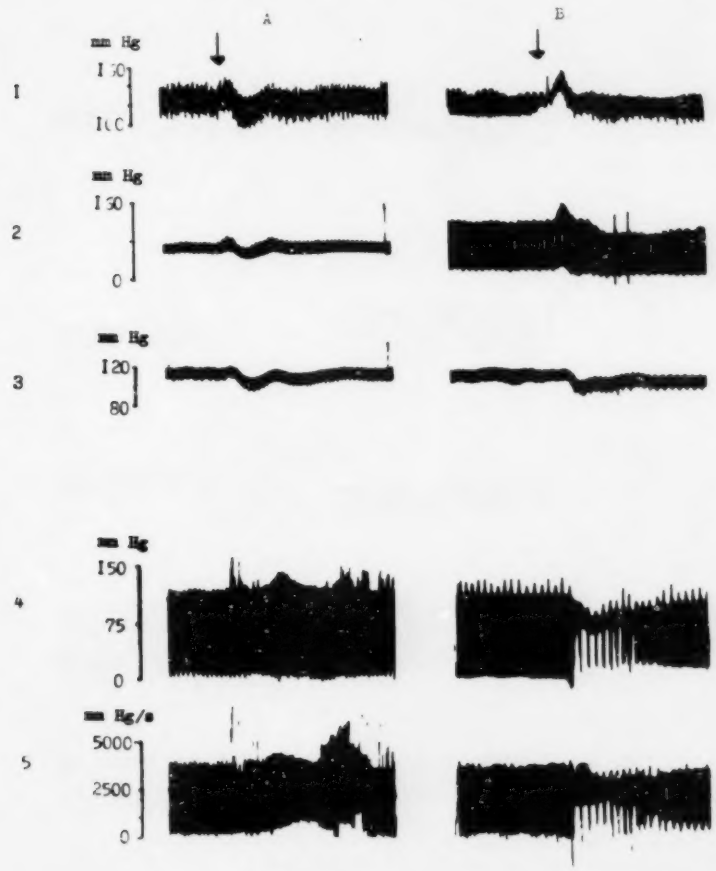

FIG. 1. Effect of intracoronary administration of epinephrine (0.1 μg) upon the tone of coronary vessels and haemodynamic indices in dogs of various age.
1—perfusion pressure in coronary artery;
2—systemic arterial blood pressure;
3—perfusion pressure in femoral artery;
4—left ventricular pressure;
5—maximal rate of increase of intaventricular pressure;
A—adult dog (2 yr); B—old dog (15 yr).
Arrow shows hormone administration.

An analysis of the ECG changes indicates that the administration of vasopressin triggers various disorders in the functions of automatism, excitability and conduction of the heart. Sinus bradycardia developed upon administration of the hormone in 100% of the cases in animals of both age groups. However, the degree to which it was pronounced in senile specimens was statistically less significant than in the mature group. For example, the administration of 0.02 U/100 g of vasopressin led to a delay in the sinus rhythm in senile specimens was 22.9 ± 1.3%, in mature specimens 29.2 ± 1.8%. Boosting the dose of the hormone to 0.2 U/100 g reveals even more pronounced age-group differences: in senile specimens 28.3 ± 1.1%, in the mature group 41.0 ± 8.4%. Disorders in the functions

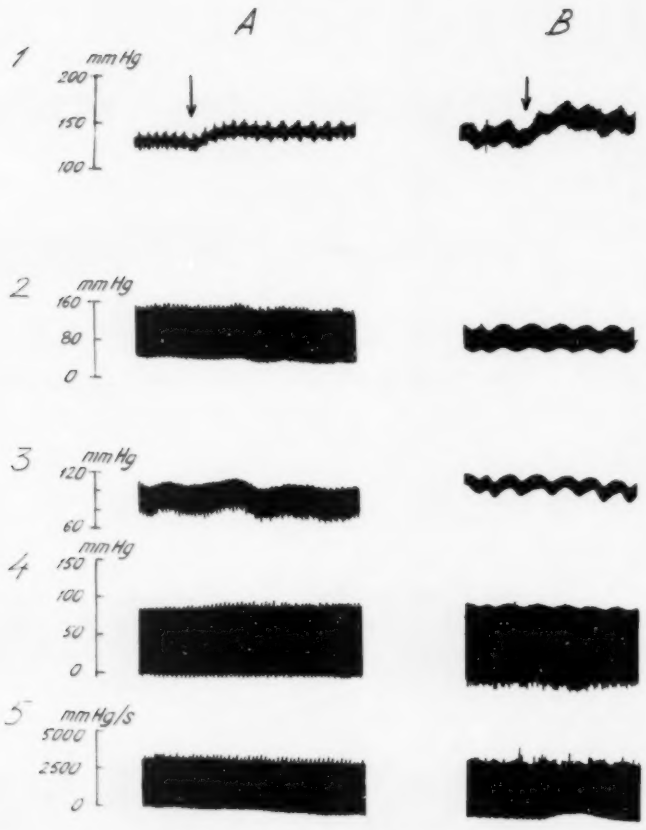

FIG. 2. Effect of intracoronary administration of vasopressin (0.05 U) upon the tone of coronary vessels and haemodynamic indices in dogs of various age.
1—perfusion pressure in coronary artery;
2—systemic arterial blood pressure;
3—perfusion pressure in femoral artery;
4—left ventricular pressure;
5—maximal rate of increase of intraventricular pressure;
A—adult dog (2 yr); B—old dog (15 yr).
Arrow shows hormone administration.

of the sinus node, upon administration of vasopressin, are also manifest in the development of sinus arrhythmia and wandering pacemaker. Sinus arrhythmia is observed primarily in mature animals. Even in small dosages, it was possible to frequently observe migration of the rhythm pacemaker in senile specimens, the pacemaker may be the atrioventricular node and even centres of the third order (idioventricular rhythm). In mature specimens migration of the pacemaker is restricted to the area of the atrium. The administration of vasopressin results in atrioventricular conduction disorders. The PQ interval in senile specimens increases from 58.2 ± 2.2 to 97.5 ± 2.5 ms, in mature specimens from 53.3 ± 1.6 to 70.1 ± 1.4 ms. Transversal heart blocks are more frequently observed in senile specimens. Consequently, it may be said that in senile animals, vasopressin leads to more pronounced changes in the phase structure of the heart's action, haemodynamic

indices, functions of contractility, excitability and automatism. In senility, the coronary vessels are more sensitive to vasopressin. For instance, an intracoronary administration of 0.05 U of vasopressin in senile dogs results in a rise in perfusion pressure in that vessel of $+25.3 \pm 2.6$ mm Hg and $+13.3 \pm 1.3$ mm Hg in mature dogs (Fig. 2). The rise in the tone of coronary vessels in senile dogs was accompanied by more pronounced changes in the contractile ability of the myocardium.

It has been established that tachyphylaxis phenomena develop under the action of vasopressin on the cardiovascular system (Nash, 1965; Abinder *et al.*, 1971). It appears that every subsequent administration of hormone triggers a lesser effect, i.e. acting upon the cardiovascular system, vasopressin makes it resistant, for a period of time, to repeated action. It would seem, however, that this phenomenon would be weaker as age increases.

A comparison was carried out of the electrocardiographic changes upon the first and second administrations, in 20 min, of vasopressin (0.1 U/100 g of body weight) in mature and senile rats. From Fig. 3, it is evident that upon the first administration of the hormone, the amplitude of the T wave changes by $66.4 \pm 11.1\%$, and after the second administration only $20.2 \pm 4.76\%$. In senile specimens, the index after the first administration changes by $99.5 \pm 21.4\%$, whereupon the second administration results in a change of $49.9 \pm 11.4\%$.

Vol. 16 1981

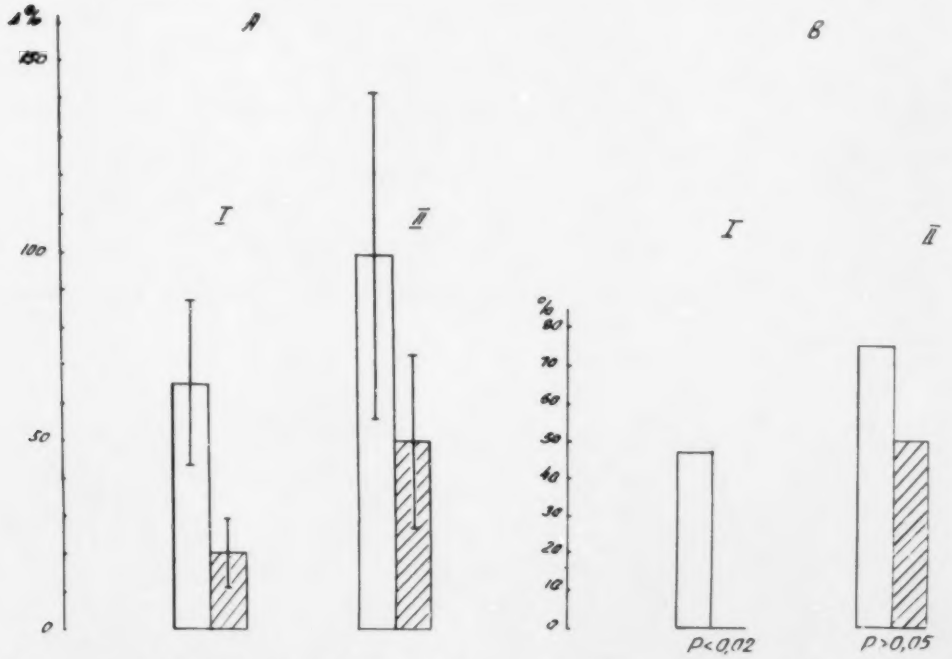

FIG. 3. Changes in amplitude of T wave (A) and occurrence of arrhythmias (B) at single and repeated (after 20 min) administration of vasopressin to adult and old rats.
I—adult animals; II—old animals;
white columns—single administration;
dashed columns—repeated administration.

The incidence of different types of arrhythmias also testifies to lesser tachyphylaxis in senility. For example, if, in senile animals, a second injection of vasopressin, in all

instances, leads to no arrhythmias (upon the first injection it is observed in 46.2% of the cases), then in senile animals arrhythmias after the second injection, are observed in half of the experiments.

Insulin

The immunoreactive insulin content in the blood of senile animals as compared to mature specimens grows at almost a double rate, from 28.4 ± 1.2 μU/ml to 53.0 ± 3.6 μU/ml. However, the total insulin-like activity in blood, determined biologically, drops from 3.9 ± 0.9 (mature) to 2.0 ± 0.1 μg of glucose g/3 h (senile). This discrepancy between the total content of insulin and insulin-like activity of blood was explained by us by the fact that substances inhibiting the activity of that hormone accumulate in the blood in old age.

The administration of insulin, as is evident from Table 3, leads to marked haemodynamic changes—a statistically significant drop in minute volume, cardiac index, the output index

TABLE 2. INFLUENCE OF VASOPRESSIN ON HAEMODYNAMICS IN MALE RATS OF DIFFERENT AGE (MEAN VALUE ± STANDARD ERROR)

Indices	Adult		Old	
	Control	Vasopressin 20 mE/100 g	Control	Vasopressin 20 mE/100 g
Systemic arterial pressure (mm Hg)	89.4 ± 1.7	+74.6 ± 4.2	91.4 ± 3.0	+63.3 ± 2.8
Cardiac output (ml/min)	91.7 ± 3.6	−15.9 ± 1.8	75.8 ± 3.7	−28.3 ± 2.9
Heart rate (beats/min)	445.6 ± 8.4	−127.8 ± 7.3	448.0 ± 11.2	−88.0 ± 6.9
Stroke volume (ml)	0.206 ± 0.007	+0.031 ± 0.003	0.169 ± 0.007	−0.040 ± 0.005
Cardiac index (1/min/m^2)	1.62 ± 0.05	−0.24 ± 0.03	1.19 ± 0.04	−0.43 ± 0.005
Left ventricular minute work index (kg/m^2)	1.94 ± 0.07	+0.09 ± 0.07	1.48 ± 0.09	+0.16 ± 0.11*
Systemic vascular resistance (dyn · s · cm^{-5})	81318 ± 3233	+100521 ± 9884	101135 ± 5462	+177666 ± 13347

Note: + increase; — decrease.
*Statistically nonsignificant, $p > 0.05$.

TABLE 3. INFLUENCE OF INSULIN ON HAEMODYNAMICS IN RATS OF DIFFERENT AGE (MEAN VALUE ± STANDARD ERROR)

Indices	Adult		Old	
	Control	Insulin 0.15 E/100 g	Control	Insulin 0.05 E/100 g
Systemic arterial pressure (mm Hg)	79.1 ± 4.4	−17.4 ± 4.23	84.2 ± 2.6	−17.6 ± 5.1
Cardiac output (ml/min)	92.8 ± 4.27	−14.3 ± 3.1	76.2 ± 2.4	−22.5 ± 5.0
Heart rate (beats/min)	364 ± 22	−52.4 ± 6.2	336 ± 8.3	−53.1 ± 10.6
Stroke volume (ml)	0.25 ± 0.01	−0.04 ± 0.01*	0.21 ± 0.02	−0.03 ± 0.01*
Cardiac index (1/min/m^2)	1.986 ± 0.04	−0.480 ± 0.05	1.348 ± 0.03	−0.408 ± 0.04
Left ventricular minute work index (kg/m^2)	2.094 ± 0.02	−0.914 ± 0.06	1.569 ± 0.03	−0.652 ± 0.06
Systemic vascular resistance (dyn · s · cm^{-5})	69416 ± 3724	+7934 ± 2910*	90811 ± 9063	+11666 ± 2716*

Note: + increase; — decrease.
*Statistically nonsignificant, $p > 0.05$.

of the left ventricle, a drop in the arterial pressure system and a slower rhythm of heart contractions. Therefore, it is possible to assert that in the senile specimens the minute volume of blood decreased by 22.5 ± 5.0 ml/min, in mature specimens by 14.3 ± 3.1 ml/min. The cardiac index fell correspondingly by 0.480 ± 0.05 and 0.408 ± 0.041 ml/min/m^2. The increase in the total peripheral resistance of the vessels were 11666 ± 2716 dyn · s · cm^{-5} in old rats and 7934 ± 2910 in adult rats. The changes in these parameters in senile animals were observed upon the administration of 0.05 U/100 g of hormone, and in mature animals 0.15 U/100. One may assume that the haemodynamic changes described above are related to the direct action of insulin and are not due to hypoglycemic effect. The fact that haemodynamic changes typical of insulin remain upon the simultaneous administration of insulin and glucose testifies to this. Consequently, the administration of small doses of insulin leads to substantial haemodynamic changes, what is more, they are pronounced to a greater degree in senile specimens.

Estradiol-dipropionate

Smaller doses of estradiol-dipropionate cause haemodynamic changes in senile animals. For example, the administration of 0.3 mg/100 g of hormone intraperitoneally in senile animals, in a matter of 15–20 min, leads to the development of a statistically significant increase in the minute volume (by 16.3 ± 2.1 ml/min), cardiac index (by 0.439 ± 0.041 l/min/m^2), output index of the left ventricle (by 0.649 ± 0.03 ml/m^2/min). Statistically significant haemodynamic changes develop in mature specimens upon administration of a dose of 0.5 mg/100 g. The tendency of this reaction and the actual degree of the change in them corresponds to the changes in senile specimens.

Thyroxin

Our data seem to indicate that with age there are irregular changes in the content of total and free thyroxin in the blood. For instance, the total thyroxin content in senility seems to decrease (from 5.5 ± 0.7 in mature to 2.1 ± 0.21 μg/100 ml in senile specimens). However, there is practically no change in the level of free thyroxin: in mature rats 4.3 ± 1.2 mg%, in senile 3.99 ± 0.31. As was pointed out earlier, this is due to the drop in the thyroxin-binding ability of the proteins in blood serum. After 4 days of administering thyroxin (10 μg/100 g intraperitoneally), there is a pronounced increase in the haemodynamic changes and the contractile ability of the myocardium, moreover, in the senile animals this was more significant than in mature animals (Table 4). In this period, the minute volume of blood in senile specimens increased by 255%, while in mature animals it increased by 104%. The output index of the left ventricle went up correspondingly by 405 and 255%. The maximum rate of build-up of intraventricular pressure increased in senile rats by 91%, in mature rats by 33%. After a fortnight of thyroxin injections the mature animals were found to have an increasing minute volume of blood, output index of the left ventricle, pressure in the left ventricle, maximum rate of build-up of intraventricular pressure, maximum speed of myocardial fiber contractions. In the senile specimens, these indices, compared to the 4 day injections, begin to go downwards. For example, in senile animals the increment in minute volume on the 14th day of thyroxin administration as compared to the 4th day was 71% lower, the increment in the cardiac index by 68%, the increment in the output index of the left ventricle by 122%, and the change in maximum rate of build-up of intraventricular pressure by 18%.

Vol. 16 1981

TABLE 4. INFLUENCE OF THYROXIN ON HAEMODYNAMICS AND CONTRACTILE FUNCTION OF THE MYOCARDIUM IN RATS OF DIFFERENT AGE (MEAN VALUE ± STANDARD ERROR)

Indices	Adult: Control	Adult: Thyroxin 10.0 mg/100 g 4th day	Adult: Thyroxin 10.0 mg/100 g 14th day	Old: Control	Old: Thyroxin 10.0 mg/100 g 4th day	Old: Thyroxin 10.0 mg/100 g 14th day
Systemic arterial pressure (mm Hg)	79.7 ± 3.2	+ 38.4 ± 3.5	+ 50.6 ± 11.0	84.2 ± 2.6	+ 35.8 ± 3.1	+ 28.4 ± 6.2
Cardiac output (ml/min)	92.6 ± 3.0	+ 130.6 ± 8.5	+ 215.2 ± 13.9	76.2 ± 2.4	+ 194.6 ± 12.3	+ 140.4 ± 9.7
Heart rate (beats/min)	349 ± 21	+ 109 ± 7.3	+ 240 ± 21	335 ± 10	+ 202 ± 8.4	+ 201 ± 17
Stroke volume (ml)	0.26 ± 0.02	+ 0.22 ± 0.01	+ 0.25 ± 0.02	0.22 ± 0.02	+ 0.29 ± 0.01	+ 0.18 ± 0.02
Cardiac index (l/min/m^2)	1.890 ± 0.02	+ 2.638 ± 0.03	+ 4.355 ± 0.03	1.342 ± 0.06	+ 3.293 ± 0.05	+ 2.376 ± 0.02
Left ventricular minute work index (kg/m^2)	2.016 ± 0.13	+ 5.169 ± 0.20	+ 8.963 ± 0.04	1.533 ± 0.03	+ 6.209 ± 0.15	+ 4.352 ± 0.02
Systemic vascular resistance (dyn · s · cm^{-5})	70360 ± 8319	− 27693 ± 7959	− 35484 ± 7520	90757 ± 10512	− 55331 ± 8612	− 49035 ± 6386
Left ventricular pressure (mm Hg)	89.7 ± 3.1	+ 42.6 ± 4.0	+ 46.1 ± 4.9	92.0 ± 3.0	+ 40.9 ± 3.8	+ 24.0 ± 3.6
Maximal rate of rise of left ventricular pressure (mm Hg/s)	3378 ± 250	+ 1120 ± 231	+ 1186 ± 242	2650 ± 189	+ 2420 ± 312	+ 561 ± 148*
Contractility index (Relative Units)	60.0 ± 2.3	+ 11.9 ± 0.2	+ 16.8 ± 2.0	47.4 ± 3.9	+ 15.1 ± 0.4	+ 4.7 ± 0.9*

Note: + increase; − decrease.
*Statistical nonsignificant, $p > 0.05$.

DISCUSSION

The most intimate aspects of the activity of cells, including the activity of their genetic apparatus are subject to hormonal control. It may be declared that the basal level of regulating the activity of the heart depends, to a considerable degree, on a whole set of hormonal actions. Changes in secretion, hormone metabolism and the state of cellular receptors represent one of the causes of the changes in metabolism and functions of the cardiovascular system in senility.

A reduction in the effective concentrations of a number of anabolic hormones (insulin, sex hormones) promotes the development of insufficient plasticity in ensuring functions in old age. It should be recalled that according to Meyerson (1967), in animals subjected to alloxan diabetes or hypophysectomy, coarctation of the aorta did not result, or if it did, then only insignificant hypertrophy of the heart. In senile animals, hypertrophy of the myocardium, upon coarctation of the aorta is less pronounced, and they rapidly develop heart insufficiency (Frolkis *et al.*, 1977). In all likelihood, the change in "hormonal support" of the heart's metabolism in old age has definite meaning here.

Vol. 16 1981

The relative growth in epinephrine content and the decrease in content of norepinephrine can, to a certain extent, promote a redistribution of the reaction of the heart and vessels in the overall haemodynamic response. We have in mind works which indicate that epinephrine has a greater impact on α-adrenoreceptors, while norepinephrine has a greater effect on β-adrenoreceptors.

We belive that the increase in vasopressin content, which causes spasms of the coronary vessels, is quite important in changing the reaction of the cardiovascular system in old age, in developing coronary bloodflow disorders. A combination of lowering the effective concentrations of anabolic hormones and boosting the overall concentration of hormones inducing intense energetic metabolism (epinephrine, vasopressin) may be an important metabolic mechanism for reducing the heart's capacity to adapt to old age.

It is self-evident that the reaction of any organ, the heart included, depends not only on the hormone content in the blood, but also on the sensitivity of the tissues to their action. In this study, thanks to the use of hormones in a wide range of doses, a comparison was made between the sensitivity of the cardiovascular system (determined by threshold concentrations of substances that trigger statistically significant changes) and its reaction capacity (possible range of reactions while increasing concentrations of hormones). Such a comparison is all the more interesting because it seems to model the situation of those hormonal changes which inevitably occur while the organism is working.

It was shown that in senility, the heart becomes more sensitive to the action of a number of hormones—epinephrine, vasopressin, insulin, estradiol-dipropionate, thyroxin. Upon the administration of small doses of these hormones, the more pronounced haemodynamic reactions, change in the contractility of the myocardium, ECG and others were observed in senile animals. However, the heart's ability to react to the action of hormones falls in old age. This confirmed by the fact that upon increasing the concentrations of hormones, the possible amplitude of the increment of the minute volume of blood, arterial pressure, the myocardial contractile index in mature animals is greater than in senile specimens. A comparison of the sensitivity and the ability to respond to the action of hormones on the heart leads to the conclusion that the possible range of its reactions decreases in old age. More than that, under prolonged administration of hormones, the senile animals may develop a qualitatively new reaction. For instance, during a fortnight's administration of thyroxin, in mature specimens it was possible to observe a rise in the indices for myo-

cardial contraction and haemodynamics, while in senile specimens, they begin to dip downwards. The heart's increased sensitivity to a number of hormones (insulin, estradiol-dipropionate, thyroxin) when their concentrations are low, is of definite adaptive significance for maintaining a basal hormonal level in regulation of the heart. At the same time, a drop in the response ability limits the possible range of the myocardium's reactions. It can only be regretted that most investigators who study the age peculiarities of reactions, make use only of several doses of physiologically active substances which are administered, and that is why they are unable to grasp a proper picture of the changes in the response of the cardiovascular system.

A significant role in changes of the heart's functions in senility, in the development of its pathology is played by the changes in the content of vasopressin, cathecholamines and the sensitivity of the cardiovascular system to them. A growth in the content of vasopressin in the blood, in conditions when the vessels are highly sensitive to it, and weakening taxiphylaxis, according to our findings, represent one of the factors that leads to arterial hypertrophy and coronary insufficiency in old age. Disorders in the functions of the heart in senility are promoted by a rise in the content of epinephrine in the blood, and its pronounced action on the myocardium.

Increasing the heart's sensitivity to hormones in old age, and reducing its ability to respond, leads to the point where the reaction of the cardiovascular system while growing old acquires a long, protracted course. Thus, irregular changes in the concentrations of hormones in the blood, changes in sensitivity and the myocardium's ability to respond to their action leads to changes in the hormonal regulation of the heart, to the development of aging metabolic and functional changes.

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BIOLOGICAL AGE AND ITS ESTIMATION. I. STUDIES OF SOME PHYSIOLOGICAL PARAMETERS IN ALBINO RATS AND THEIR VALIDITY AS BIOLOGICAL AGE TESTS

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Vol.
16
1981

INTRODUCTION

RELIABLE and objective estimation of biological or functional age of man is recognized to be one of the most important problems of gerontology (Bourliere, 1970; Comfort, 1969, 1972; Chebotarev, 1972; Shock, 1978). It is necessary for establishing individual "physiological" age and for checking the efficiency of any efforts undertaken to weaken the manifestation of aging.

The workers concerned with experimental studies of lifespan prolongation need to estimate the biological age of laboratory animals. Usually in such experiments the survival and mortality data suffice, but these data contain no information on the mode of action of the agent studied on the physiological parameters; observation of the animal mortality even with rodents takes three to four years; finally, such investigations require many animals, at least 40–50 in the treated and untreated groups.

Determination of biological age of laboratory animals as a method of estimating the efficiency of interference with aging offers some advantages. This does not require many years' observations over a great number of animals, being based on measuring some physiological parameters, and it provides information on the action of the agent studied on different systems and the whole organism. The mortality data make it possible to estimate total population aging whereas from the biological age the individual aging rate can be inferred.

We consider some physiological and biochemical parameters in albino rats regarding their usefulness as tests for estimating biological age. It seems necessary to give first of all the criteria of usefulness of each individual test and to discuss which indices should be included into a test battery.

Criteria for each test

The requirements are in principle the same as those developed earlier for human biological age estimation (Bourliere, 1970; Comfort, 1969).

1. Directness and regularity of age-related changes. Parameters which change during life in one direction (increase or decrease) may be thought of as the best for measuring the biological age. It is of special value if they change linearly or exponentially.

2. Correlation with the chronological age. It seems desirable to select the tests which closely correlate with the chronological age either directly or after transformation.

3. The rate of change. It may be useful that the indices selected would change with age quite intensely, i.e. the differences between the successive age groups could be detectable.

4. Treatment insensitivity. In the cases when the biological age tests are supposed to

be used for comparison of the aging rates in two groups, either being treated with a certain agent, there arises an additional requirement of applicability of the tests, i.e. the treatment insensitivity criterion.

Moreover, the author's experience with rats allows them to suggest the following additional criteria:

5. Absence of appreciable seasonal variations, which are found, for example, when studying the activity of some enzymes.

6. Mutual compatibility of the tests. For example, if for determining the primary immune response an animal undergoes immunization, then simultaneous determination of even such parameters as heart weight or aortic collagen, not speaking of the enzyme activity, may be affected.

7. Simplicity and accessibility.

The indices to be included in a test battery

It is impossible to estimate correctly the biological age of an individual with the aid of one parameter alone. This estimate will be incorrect at least because of the two reasons:

(1) a low accuracy which may generally be achieved in measuring the individual age using a biological parameter (Young and Rickert, 1973);

(2) unequal aging rates of different functions and systems resulting in a divergence between the biological age estimates obtained with the corresponding parameters (Dubina and Razumovich, 1975).

The question of how many and which particular tests should be used for biological age estimation in animals remains to be solved.

The biological age of an organism as a whole may be thought of as involving a great number of components, every one being a "biological age" of the corresponding physiological function or system.

If out of an infinite number of physiological functions one takes a finite number of those which are the most important for aging, then the degree of their age-related changes gives an idea (with some approximation) about the degree of aging of the whole organism.

It is apparent that the choice of these most significant and essential functions and the corresponding indices may be based on considerations both of their relation to "primary" aging and of their destructiveness.

According to Cutler (1976), "the basic concept of primary aging processes is the hypothesis that many of the age-dependent dysfunctions at the phenotypic level are a part of a highly complex and interdependent process which is initiated or caused by fewer or less complicated dysfunctions at a more fundamental level". In other words, such age-related changes at a molecular level which regularly result from the action of time on living systems and for which all the other age-related changes may be considered as their consequences are meant to be primary aging processes.

The question which aging processes are primary is disputable. Some age-dependent dysfunctions which arise in the cell nuclei, long-lived proteins and cell membranes, e.g. accumulation of cross-links in nucleoproteins (Asharia *et al.*, 1972; Cutler, 1976), lipoperoxidation (Tappel, 1968), progressive cross-linking in collagen and elastine (La Bella and Paul, 1965) may probably be thought of as primary aging processes. Some direct molecular consequences of these dysfunctions such as lipofuscin accumulation (Strehler *et al.*, 1959), a decreased template capacity of chromatin (Zhelabovskaja and Berdyshev, 1972), and an age-related rise of the number of altered enzyme molecules

(Gershon and Gershon, 1970; Holliday and Tarrant, 1972) might also be possibly considered as primary aging processes.

Not all of these are practical tests for biological age estimation because they are bulky and labour-consuming. On the other hand, some secondary processes which correlate fairly well with "primary" changes and are easily measurable and consistent with the criteria listed above, can be treated as measures for the primary processes.

Destructiveness is recognized as one of the four criteria of the true aging processes (Strehler *et al.*, 1959).

While selecting the parameters for biological age estimation, such a property as destructiveness seems quite attractive. Indeed, a contribution of the processes represented by such indices to an age-dependent decrease of the organism adaptability and to an increase of the death probability is evident.

If biological age tests are to measure destructiveness, then the test battery should be supplemented with tests which reflect a decline of the main vital functions such as those of immune, endocrine, cardiovascular, nervous systems, energy and lipid metabolism, etc., along with "nondestructive" indices such as hair greying. According to Hollingworth *et al.* (1965) the age-correlation coefficient for hair greying is +0.717, while for "destructive" index systolic pressure it is +0.519.

Vol. 16 1981

Consideration of the tests chosen for the present study

The indices chosen for the study of the age-dependent changes in rats characterize either the status of some vital functions (a cardiovascular system, a connective tissue system, oxidation–reduction reactions) or some fundamental processes which are recognized as primary or which are directly associated with the primary aging processes (collagen cross-linkage, altered protein synthesis, a decrease of the amount of functional units).

Allometric indices. According to Shock (1962), the "active protoplasm mass" regularly declines with age. This idea is based on observations of a loss with age of functional units by some organs such as kidneys, brain, muscles. The cell loss may be determined directly from the total potassium content in a body (based on ^{40}K), since potassium is an intracellular element (Anderson and Langham, 1959), and indirectly from lowering of the absolute or relative weight of some organs, particularly, a thigh muscle and musculus gastrocnemius (Yengst *et al.*, 1959; Barrows *et al.*, 1962). A decrease of the muscle mass is essential for a progressive decline of the working capacity with age.

Some organs during ontogenesis become involved in counterprocesses: alongside with a loss of functional units, the remaining units become hypertrophic and connective tissue increases. This can result in an increase of an organ weight with age. The heart weight of albino rats was shown to increase progressively with age (Nagornyi *et al.*, 1963).

Body weight is sometimes included in test batteries for estimating the biological age of man (Furukawa *et al.*, 1975; Shock, 1978). Body weight of rats increases in the first half of life, then remains constant and falls at very old ages, the time of each period being unequal for different races (Hrachovec and Rockstein, 1962). The rat body weight is likely to be a useful test for biological age estimation. Whereas a decreased weight in caloric-restricted rats is accompanied by an appreciable lengthening of the lifespan, in normal rats "contrary to what one might believe, there is a very positive correlation between size of body attained and length of life" (McCay *et al.*, 1956).

In the present work age-related changes in weights of body, gastrocnemius and heart of rats are considered.

Indices of the cardiovascular functioning. The age-dependent changes of the cardiovascular system certainly contribute to the general aging of mammals. It is generally recognized that in ageing mammals the cardiac output decreases, the blood pressure rises and the cardiac rhythm becomes slower. The last two indices are usually included in test batteries for determining the biological age of man (Mintz, 1978; Shock, 1978). Rhythm measurements (from ECG) were included in a test programme for estimating the biological age of rats (Hofecker, 1976). We have measured all the above indices.

Indices of the connective tissue status. An increased amount of inter- and intramolecular bonds in collagen is assumed to result in a change of its physical and chemical parameters. The following indices are suggested to be measured for estimation of biological age: "labile" collagen yield into physiological solution (Verzar, 1964), mechanical strength of fibres (Everitt *et al.*, 1968), fluorescence intensity of tendon collagen (La Bella and Paul, 1965), rate of collagen digestion by collagenase (Hamlin and Kohn, 1971). In the present study labile collagen yield was used as a test.

During life the physical and chemical properties of collagen change and its content in some tissues increases. Cardiac muscle collagen contents were used to compare the aging of intact rats with those forced to chronic exercise (Tomanek *et al.*, 1972). On the other hand, total skin collagen content in rats increases intensely only in the growth period (Moroz, 1969). The data on age-related changes of aortic collagen contents in rats are contradictory (Georgi *et al.*, 1976).

In the present work the toal and labile skin collagen as well as total myocardial and aortic collagens in rats were measured. Aorta zinc and copper contents were also examined. It was shown that human aorta zinc (Gulko, 1966) and copper (Zinsser *et al.*, 1957) increase with age. No relevant data for rats have been found by the authors in the available literature.

Indices of oxidation–reduction processes. A decrease of energy metabolism rates is considered to be one of the most important signs of aging.

The age-dependent shifts in energy metabolism rates are sometimes estimated as activities of enzymes involved in the energy metabolism. Liver succinate dehydrogenase activity has been used as a test for estimation of biological age of rats (Hofecker, 1976).

According to Oeriu and Tigheciu (1964), age-dependent changes in oxidation processes are manifested by higher amounts of oxidized glutathione in blood and organs. An oxidized glutathione content was suggested by the authors as a test of biological age; this test proved useful for rats (Kolomiichuk, 1972; Dubina and Razumovich, 1975).

An age-dependent decrease of glutathione reductase activity in human erythrocytes was reported (Fornaini *et al.*, 1970).

In the present study, oxidized glutathione concentration in rat blood and glutathione reductase activity of rat liver were examined.

Indices which indicate occurrence of altered proteins. According to Orgel's (1963) hypothesis the protein-synthesizing apparatus of a cell is held to become less accurate with age which results in production of mis-translated proteins with abnormal sequences. Holliday and Tarrant (1972) used an increased thermolability of the altered proteins to support Orgel's hypothesis. They demonstrated that in ageing human fibroblast cultures (as the doubling number increases), the heat-labile fraction of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase grows.

If Orgel's hypothesis is correct, the heat-labile enzyme fraction should grow not only in aging cell cultures but during aging of an organism. Indeed, thermolability of glutathione reductase in lenses of old men was found to increase (Harding, 1973).

In the present work the thermolability of glucose-6-phosphate dehydrogenase of rat liver was studied.

MATERIALS AND METHODS

The studies were performed with Wistar rats (non-inbred), males and females, at the age between 5 and 29 months. The animals which had fasted were killed by decapitation, after haemodynamic assay in some cases or without it.

Indices characterizing the cardiovascular status

They were determined under ether–hexanal anesthesia. The cardiac output (*CO*) was assayed with the thermodilution technique (Fegler, 1954; Gurevich *et al.*, 1967). The intravascular temperature in the left carotid was measured with a microresistor MT-54 mounted into a metal catheter. The recording was performed on an electronic self-recorder potentiometer EMP-09. Physiological solution, 0.2 ml in volume, injected at the junction of the vena cava superior into the right auricle with the aid of a polyethylene catheter was used as an indicator. *CO* was calculated from the thermodilution curve.

A heart rate (*HR*) was calculated from the electrocardiography data recorded with needle intracutaneous electrodes. The *CO* values normalized on unit body weight were used to calculate a blood circulation index (*BCI*). Mean blood pressure (*BP*) was measured in the right carotid with an electrical manometer EM-2-01 mounted into a catheter. In each experiment *CO*, *HR* and *BP* were measured 3–5 times with 5–10 min intervals and the means were evaluated.

Vol. 16 1981

Allometric indices

The heart was removed, washed of blood and deprived of the pericardium. Large vessels were cut from close to the surface and the heart was desiccated with a filter and weighed. Gastrocnemius muscle was isolated from the left leg, deprived of fat and fascia, washed, desiccated with filter paper and weighed quickly. Relative weights of muscle gastrocnemius and heart were calculated (%) based on the animal weight before killing.

Tissue collagen content

Some part of a cardiac muscle (~0.2 g) from the free wall of the left ventricle (without papillary muscles and tendons) was crushed, placed into an ampoule and dried to a constant weight. The aorta was isolated from the initial arc to the bifucation, deprived of the surrounding tissue, washed, desiccated with a filter, put into an ampoule and weighed. The back skin was carefully cleaned of hair with a blade, deprived of subcutaneous fat and rubbed with ether. The skin was crushed with shears into 1 × 1 mm pieces, stirred, and an aliquot of 0.2 g was placed into an ampoule. One aliquot was used for determination of the dry weight. All the ampoules were filled with 6N HCl, 2–4 ml, sealed and heated at 115°C for 7 h. The hydrolysate was filtered, neutralized and analyzed for oxyproline with Neuman and Logan's (1950) method, a modification of Zaides *et al.* (1964). The heart, skin and aorta oxyproline contents were multiplied by 7.46 to convert into the collagen basis. Skin and heart collagens were calculated on a dry weight basis; aortic collagen was based on fresh weight.

Labile skin collagen was determined according to Verzar (1964). An aliquot (1.0 g) of the crushed skin was incubated for 10 min in 0.15 M NaCl solution at 65°C and the filtrate was lyophylized. The lyophylisates were kept at −4°C. In order to assay the oxyproline content, they were dissolved in 6N HCl, transferred to ampoules, and the collagen content was further determined as above.

Aortic zinc and copper contents

The aorta (isolated in the same way as for collagen analysis) was placed into a platinum crucible and ashed at 500°C with one drop of concentrated HNO_3. The ash was dissolved in 0.5 ml of 6N HCl and diluted to volume with water, acetone and isobutyl alcohol (according to Allan, 1961). The aorta metal contents were assayed with the atomic absorption spectrophotometry technique on a spectrophotometer SFPA-2 on a fresh weight basis. All the precautions were taken in the metal assays just as in trace element analysis.

Blood oxidized glutathione

After decapitation of the animal, blood was gathered into heparinized polyethylene tube. Blood oxidized glutathione was assayed following the method of Woodward and Fry (1932). A blood volume of 3 ml was added to 24 ml of water and after haemolysis of the blood and protein precipitation by 25% sulphasalicylic acid the mixture was filtered. The reduced glutathione was assayed in the filtrate aliquot by iodometric titration with 1 or 2 drops of starch. The remaining filtrate was supplemented with powdered zinc to reduce oxidized glutathione and the reduced glutathione content was measured again, the reduced glutathione amount in this case corresponding to the total blood glutathione. Oxidized glutathione (mg %) was calculated as the difference between the total and reduced glutathione.

Liver glutathione reductase (EC 1.6.2.4)

The liver was isolated and washed with cold 0.1M potassium phosphate buffer (pH 7.5) with 0.002M EDTA. The homogenate (1 : 10) was prepared in the same buffer, and was then centrifuged at 2,000 ***g*** for 20 min. The glutathione reductase activity of the supernatant was assayed in the presence of reduced glutathione and $NADP \cdot H_2$ from the change of the optical density at 340 nm for 3 min (Wendell, 1968). The measurements were performed on a spectrophotometer SF-4 and the results were expressed in conventional units (D/mg protein). The protein was assayed following Lowry *et al.* (1951)

Thermolability of liver glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

Liver homogenate (1 : 10) prepared on 0.15M KCl was cold centrifuged at 2,000 ***g*** for 20 min. An aliquot of the supernatant was placed on a water bath (48°C) for 5 min. The glucose-6-phosphate dehydrogenase activity was assayed in the native and heated supernatant following Zakhariin's (1967) method. The incubation mixture, containing 1 ml of 0.3M tris buffer (pH 8.5), 0.2 mM $MgSO_4$, 0.4 μM NADP and 0.2 ml of the supernatant, was added to 8 μM sodium glucose-6-phosphate. After 15 min incubation at 37°C the reaction was stopped by addition of NaOH. After centrifugation the supernatant absorbance at 340 nm was measured on a spectrophotometer SF-4. The enzyme thermolability was inferred from the residual activity expressed as D_{340} heated supernatant/D_{340} native supernatant percentage. The heating conditions were chosen in preliminary study.

RESULTS

The results obtained were gathered in four age groups. The first group incorporate animals aged 5–9 months inclusive, the second, third and fourth groups include rats of 10–16, 17–23 and 24–29 months, respectively. The average values of the indices for each of the age group are presented in Tables 1 (males) and 2 (females).

The average body weight (*BW*) of the males increases markedly up to 23 months, stabilizing thereafter. The average gastrocnemius weight (*MGW*) of the males up to 9 months is somewhat lower than that of the older animals but these differences are statistically insignificant. The relative musculus gastrocnemius weight (*RMGW*) of the males decreases gradually with age. Conversely, the average weight of heart (*HW*) of male rats increases progressively with age. The *HW* rise is, however, behind the total weight growth, and the relative heart weight (*RHW*) therefore falls continuously and reaches a minimal level in rats at 17–23 months. For the males of the oldest age group *RHW* is higher and equals the value found for rats of 10–16 months.

The age-dependent changes of the averages of heart functioning are similar to those of *RHW* as regards their mode of changing and direction. Both the minute cardiac output (*CO*) and the blood circulation index (*BCI*) decrease with age, reaching a minimum at 17–23 months, and for the animals older than 24 months these quantities approach the value generally found for rats of 10–16 months.

Blood pressure (*BP*) of the males older than 17 months somewhat exceeds that of the younger animals, but this parameter in the present study was highly variable and no reliable differences between the groups could be detected. The heart rate (*HR*) of the males declines continuously with age.

The heart collagen content (*HC*), the aorta Cu content (*ACu*), the blood oxidized glutathione (*OG*) of males also exhibit marked age-related changes. *ACu* declines progressively, whereas the mode of *HC* and *OG* changes (but not the direction of the changes) is similar to that of *RHW, CO* and *BCI* since *HC* and *OG,* increasing with age, reach their maximum in rats of 17–23 months. These two variables in the oldest animals become lower again approaching the values found in the animals of 10–16 months.

We have not found any essentially age-dependent changes in the aorta Zn content (*AZn*) and in the liver glutathione reductase activity (*LGR*) in the male rats.

In the females (Table 2) *BW* increases gradually with age, this increase being more intensive in the first half life. The same may be said about *MGW*. *RMGW* of the females

Vol. 16 1981

TABLE 1. AVERAGE PHYSIOLOGICAL INDICES OF THE MALE RATS

Indices	Age groups: 5–9 months M ± se		10–16 months M ± se		17–23 months M ± se		24–29 months M ± se	
Body weight (g)	345 ± 14	(17)	383* ± 11	(14)	420‡ ± 14	(16)	411‡ ± 10	(13)
Musculus gastrocnemius weight (g)	2.17 ± 0.096	(16)	2.35 ± 0.091	(14)	2.29 ± 0.056	(13)	2.39 ± 0.103	(13)
Relative musculus gastrocnemius weight (%)	0.65 ± 0.011	(16)	0.61* ± 0.016	(14)	0.56‡ ± 0.016	(13)	0.55‡ ± 0.021	(13)
Heart weight (g)	1.17 ± 0.046	(17)	1.24 ± 0.041	(14)	1.31* ± 0.035	(14)	1.34† ± 0.039	(13)
Relative heart weight (%)	0.35 ± 0.007	(17)	0.33 ± 0.009	(14)	0.31† ± 0.008	(14)	0.33 ± 0.009	(13)
Cardiac output (ml/min)	100.5 ± 8.36	(8)	96.8 ± 5.17	(19)	78.5* ± 5.09	(10)	92.7 ± 5.38	(18)
Blood circulation index (ml/min g)	0.28 ± 0.020	(8)	0.27 ± 0.014	(19)	0.20† ± 0.015	(10)	0.23* ± 0.009	(18)
Heart rate (beat/min)	372 ± 11.9	(8)	348 ± 6.3	(19)	324* ± 13.0	(10)	315‡ ± 7.4	(18)
Blood pressure (mm Hg)	77.8 ± 4.80	(8)	77.8 ± 2.30	(19)	87.6 ± 4.15	(10)	81.6 ± 4.24	(18)
Heart collagen (mg/g dry weight)	22.8 ± 0.81	(10)	28.3‡ ± 0.66	(13)	29.2‡ ± 0.59	(15)	28.6‡ ± 0.70	(8)
Aortic metal content (μg/g fresh weight):								
zinc	17.2 ± 1.12	(9)	19.8 ± 0.80	(12)	17.7 ± 0.80	(9)	19.5 ± 1.31	(7)
copper	4.77 ± 0.663	(9)	3.87 ± 0.486	(12)	3.62 ± 0.407	(13)	2.71* ± 0.585	(7)
Blood oxidized glutathione (mg%)	5.37 ± 0.26	(8)	7.67† ± 0.69	(10)	8.57‡ ± 0.60	(10)	7.53‡ ± 0.47	(11)
Activity of liver glutathione reductase (ΔD/mg protein per 3 min)	0.156 ± 0.008	(16)	0.156 ± 0.009	(13)	0.165 ± 0.011	(14)	0.157 ± 0.011	(13)

Notes: Figures in brackets show the animal number.
Significance of the difference by Student's *t*-test as compared with group 5–9 months:
*$p < 0.05$.
†$p < 0.01$.
‡$p < 0.001$.

TABLE 2. AVERAGE PHYSIOLOGICAL INDICES OF FEMALE RATS

Indices	Age groups: 5–9 months M ± se		10–16 months M ± se		17–23 months M ± se		24–29 months M ± se	
Body weight (g)	244 ± 9	(10)	284‡ ± 7	(30)	307‡ ± 9	(17)	310‡ ± 13	(11)
Musculus gastrocnemius weight (g)	1.57 ± 0.050	(10)	1.72* ± 0.037	(30)	1.83† ± 0.056	(17)	1.86† ± 0.056	(11)
Relative musculus gastrocnemius weight (%)	0.65 ± 0.011	(10)	0.61† ± 0.008	(30)	0.60* ± 0.014	(17)	0.60* ± 0.016	(11)
Heart weight (g)	0.81 ± 0.028	(10)	0.96‡ ± 0.030	(30)	1.04‡ ± 0.039	(17)	1.01† ± 0.045	(11)
Relative heart weight (%)	0.33 ± 0.007	(10)	0.34 ± 0.008	(30)	0.34 ± 0.009	(17)	0.33 ± 0.006	(11)
Collagen contents:								
heart (mg/g dry weight)	22.3 ± 0.49	(10)	26.8‡ ± 0.51	(25)	28.9‡ ± 0.47	(16)	32.6‡ ± 0.60	(11)
skin (mg/g dry weight)	313 ± 35	(10)	660‡ ± 91	(21)	511‡ ± 19	(11)	545‡ ± 10	(11)
aorta (mg/g fresh weight)	97.5 ± 3.98	(9)	90.3 ± 2.09	(21)	76.8‡ ± 3.61	(11)	82.8† ± 1.69	(11)
Labile skin collagen (%)	14.9 ± 0.81	(10)	12.3* ± 0.64	(21)	10.9† ± 1.01	(11)	9.6‡ ± 0.33	(11)
Aortic metal content (μg/g fresh weight):								
zinc	26.2 ± 2.00	(9)	22.6 ± 0.99	(16)	21.3* ± 1.07	(6)	21.1 ± 1.93	(7)
copper	11.64 ± 2.070	(9)	7.45 ± 1.054	(16)	4.13† ± 0.223	(7)	4.57* ± 0.757	(8)
Thermolability of liver glucose-6-phosphate-dehydrogenase (%)	86.6 ± 3.5	(10)	84.7 ± 3.2	(21)	83.1 ± 3.5	(11)	77.6 ± 5.5	(11)

See Notes to Table 1.

Vol 16 198

declines with age, but not so markedly as that of the males. It is interesting that at a young age (5–9 months) *RMGW* values are equal for the females and the males. *HW* of female rats increases with age but remains at the same level after 23 months; *RHW* does not exhibit noticeable age-related changes.

The parameters characterizing the connective tissue state undergo the following changes in the females. *HC* continuously rises with age. Total skin collagen (*TSC*) is considerably higher for the rats older than 9 months as compared with the younger animals, but no essential differences have been found between the second, the third and the fourth age groups. Labile skin collagen (*LSC*) falls gradually with age; aortic collagen content (*AC*) also decreases with age but its minimum is found in the third age group rather than in the fourth one.

Aorta Zn and Cu contents in the females are higher than in the males, these differences being especially pronounced at the age of 5–9 months. After 9 months *AZn* of the females decreases and remains later at the same level. *ACu* of the females declines with age more considerably than *AZn* does, reaching its minimum at the age of 17–23 months.

Thermolability of the liver enzyme glucose-6-phosphate dehydrogenase (*TE*) in the present study varies greatly for different individuals, therefore, in spite of some age-related decrease of this variable the differences even between the extreme groups appeared statistically insignificant.

Vol. 16 1981

Table 3 contains the age correlation coefficients for all the parameters studied. The following age correlations are statistically reliable (a decreasing order): for the males *RMGW, HC, BW, BCI, OG, HW, CO, ACu, HR* and *RHW*; for the females *HC, ACu, LSC, AZn, TE, AC, BW, HW, MGW* and *RMGW*.

TABLE 3. CORRELATIONS BETWEEN THE PHYSIOLOGICAL INDICES STUDIED AND THE AGE

Indices	Males		Females	
	Number of rats	Correlation coefficient	Number of rats	Correlation coefficient
BW	60	0.476*	68	0.416*
MGW	56	0.178	68	0.311†
RMGW	56	−0.614*	68	−0.285†
HW	58	0.379*	68	0.363*
RHW	58	−0.255†	68	−0.004
BP	39	0.271	—	—
CO	31	−0.377†	—	—
HR	42	−0.361†	—	—
BCI	31	−0.423†	—	—
HC	38	0.569*	64	0.722*
AC	—	—	52	−0.424*
TSC	—	—	53	0.207
LSC	—	—	53	−0.560*
AZn	31	0.189	22	−0.454†
ACu	30	−0.376†	24	−0.625*
OG	49	0.391*	—	—
GR	56	0.065	—	—
TE	—	—	53	−0.431*

Note: Validity of the correlation coefficients:
*$p < 0.01$.
†$p < 0.05$.

DISCUSSION

Most of the parameters studied in rats in this work have been proved to be more or less closely correlated with age.

If the correlation with age were a single criterion for the test selection, all the indices which correlate with age (or those which correlate more closely, i.e. have $r > 0.5$) should be considered adequate for a test programme. Inclusion of the tests into such a programme necessitates that all they would be determined in each of the animals. Then the mutual compatibility criterion (the sixth of the criteria enumerated in 'Introduction') becomes of great importance. But when the tests mentioned were approached with this criterion in view, we were forced to reject the haemodynamic indices (except *HR*).

The haemodynamic variables *CO, BP* and *BCI* in rats are determined in acute experiments under anesthesia. In a preliminary study it has been found that *HC, ACu, AZn* and *HW* in the animals which underwent anesthesia and haemodynamic assay do not change as compared with the pair control (Dubina *et al.*, 1979). However, the other, more labile parameters do change essentially under these conditions. So, it has been found that the water content in myocardium is reliably higher in the male rats which underwent measurements of the haemodynamic parameters than that in intact males. It also appears that in rats which had been in acute experiments for a long time the *in vitro* determined hormonal response of epididymal adipose tissue was lower. The latter parameter determined from the lipolytic activity changes appreciably with age and may be a valuable test for biological age estimation (Chaika and Mazhul, 1976; Dubina and Zhuk, 1979). This however, is inconsistent with haemodynamic assay. It should not be excluded that anesthesia and acute experimental conditions may also affect some other labile parameters.

The index of cardiovascular functioning (*HR*) is measured from ECG and does not require anesthesia and operation. This index should therefore be included into the test list which may be useful for a test battery.

The first criterion (one-way movement of age-dependent changes) also imposes certain limitations on the tests to be chosen. Of the male age-correlated indices only *RMGW, HW, HR* and *ACu* change more or less linearly during the whole ontogenesis (see Table 1). The other parameters (*BW, RHW, CO, BCI, HC, OG*) reach the highest or the lowest values at the age of 17–23 months rather than in the oldest age group. In the females (see Table 2) the following age-correlated indices change in one direction: *BW, MGW, HC, LSC* and *TE*, whereas *HW, AC* and *ACu* change in the same way as *BW, RHW*, etc., of the males (a peak is found in the third age group).

Of the 14 parameters measured in male rats only *RMGW, HR, HW* and *ACu* may be suggested as suitable for the biological age test programme. In female rats, 12 indices were determined. Of them only *BW, MGW, HC, LSC* and *TE* may be suggested as the parameters useful for developing a test battery. Thus, such relatively high age-correlated indices as *HC* in males and *ACu* in females appear to be excluded from the list of the suitable indices.

The rejection of the indices which do not meet the first criterion restricts the number of useful indices and strongly contradicts some theoretical statements.

The type of age-related change when a minimum (or a maximum) value is observed in the pre-senile age group, followed by a kind of the "reverse development" is likely to be explained by the fact that the age-dependent changes were determined in this study in cross-sectional but not in longitudinal studies.

Cross-sectional studies are known to be a reason of such "incorrectness" of the age-dependent changes as those described above, both in man (Exton-Smith, 1970) and in animals (Lesser *et al.*, 1973). The parameters changing in such a manner seem to be closely related with survivability since the best values of these parameters ensure selection of individuals into the oldest age group. Moreover, according to Donato *et al.* (1979), when averages are obtained by cross-sectional sampling of the population all the parameters essential for survival ("age-specific biological parameters") obligatorily exhibit characteristic humps at old age, the humps which result from the effects of selective mortality. Consequently, the nonlinear hump-bearing parameters are of importance for studies of biological age.

On the other hand, a nonlinear mode of age-dependent changes of some parameter makes it less useful as a biological age test. It seems that a resolution of this contradiction is necessery both for practical aims (selection of the tests) and for developing the adequate concept of biological age.

Vol.
16
1981

SUMMARY

In Wistar male and female rats 18 allometric, physiological and biochemical parameters, which characterize age-related changes of the body weight and weights of some organs, cardiovascular system, oxidation–reduction processes, connective tissue system, loss of active protoplasm mass and altered protein synthesis have been studied. Correlation with age has been revealed for the following indices: relative musculus gastrocnemius weight, heart collagen, body weight, blood circulation index, aorta Cu content, oxidized glutathione of blood, heart weight, cardiac output, relative heart weight, heart rate (males); heart collagen, aorta Cu content, labile skin collagen, aorta Zn content, thermolability of liver glucose-6-phosphate dehydrogenase, aorta collagen, body weight, heart weight, musculus gastrocnemius weight, relative musculus gastrocnemius weight (females).

Cardiovascular indices (besides *HR*) are recognized to be unsuitable for a test battery because they are determined at acute experimental conditions under anesthesia and may interfere with some other indices.

With a value of the age-correlation coefficient and consistency of the parameters with such criteria as unidirectness of the age-dependent changes in view, the following indices have been suggested for estimation of the biological age of Wistar rats. For males these indices are: relative muscular gastrocnemius weight, heart weight, heart rate and aorta copper content. Heart collagen may probably be added to the list as it correlates highly with age though for males this index increases gradually only up to the pre-senile age, decreasing thereafter. For females these indices are gastrocnemius weight, heart collagen, labile skin collagen, thermolability of liver glucose-6-phosphate dehydrogenase and body weight. Because of its relatively close correlation with age and simplicity of determination aorta copper should probably be included into the list, although this parameter is not strictly unidirectional.

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AGING OF CHICK EMBRYO FIBROBLASTS *IN VITRO*—VI. DIFFERENT AGING PATTERNS OF POPULATION DOUBLING RATE AND POLYPLOIDIZATION*

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Vol. 16 1981

INTRODUCTION

ALTHOUGH chick embryo fibroblasts as well as human diploid fibroblasts have been utilized as a model system for studying on the phenomenon of *in vitro* aging, their aging patterns are known to be somewhat diverse (Hay and Strehler, 1967; Pontén, 1970; Lima and Macieira-Coelho, 1972). Previously, we have observed that the shapes of curves on the variations in population doubling rate throughout their lifespan fell into two types; the rate of some chick cell lines remained unchanged at the first several passages (type A: Fig. 1a), but the rate of the other lines decreased exponentially without any plateau after primary culture (type B: Fig. 1b) (Kaji and Matsuo, 1978*a*). At present, the reason for how the different aging patterns of chick cells are brought about is inexplicable.

To see whether the difference between the two aging patterns of population doubling rate is due to any artefact in culture, the variations in population doubling rate and polyploidization during their lifespan were examined using the sublines of chick cells separately obtained from individual embryos.

MATERIALS AND METHODS

Cell culture

Fertile White Leghorn eggs were obtained from the Goto Chick Farm (Nagoya, Japan). The procedures for isolation and subcultivation of chick embryo fibroblasts have been described previously (Kaji and Matsuo, 1979). Cells were grown in Temin-modified Eagle's medium (Daigo Eiyo Kagaku, Osaka, Japan) supplemented with 10% fetal bovine serum (Grand Island Biological Company, New York, NY, U.S.A.), 10% tryptose phosphate (Difco Laboratories, Detroit, MI, U.S.A.), penicillin (250 U/ml; Meiji Seika, Tokyo, Japan), and streptomycin (50 μg/ml; Meiji Seika).

Cell counts were electronically made with a Coulter Counter model D (Coulter Electronics, Hialeah, FL, U.S.A.).

Population doubling rate at each passage was determined according to the method of Kaji and Matsuo (1978*a*). The lifespan was defined to come to an end when the number of cells harvested is less two weeks after subcultivation than the number of cells inoculated.

Determination of ploidy patterns in cell populations

The ploidy patterns of the nuclei of cells left confluent for 3 days without medium renewal were analysed by the method described previously (Kaji and Matsuo, 1979) using a Fluorescence-Activated Cell Sorter III (Becton-Dickinson Electronics Laboratory, Mountain View, CA, U.S.A.) with an argon ion laser at 488 nm.

Polyploidization index

Polyploidization index (PI) has been defined by Kaji and Matsuo (1981).

$$\mathrm{PI} = \frac{\sum_{n=1}^{7} (\%\text{ of } 2^n\text{C nuclei at each population doubling level}) \times n}{100}$$

*Contribution TMIG-I No. 30.

RESULTS AND DISCUSSION

As mentioned above, the *in vitro* aging patterns of population doubling rate in chick cells are classified as type A and B. The lifespan of type A and B cells is given in Table 1, and the variations in their population doubling rate are shown in Fig. 1. Among type A (Fig. 1a) or B (Fig. 1b) sublines of cells obtained from an embryo, there was no difference in their total cumulative population doublings and in their aging patterns of population doubling rate. The findings appear to show that the difference in the aging patterns of population doubling rate designated as type A and B is not resulted from any artificial lesion in culture.

TABLE 1. THE *in vitro* LIFESPAN OF CHICK CELLS

Cell subline*	Passages	Population doublings	Lifetime (days)
A-1	16	23.0	102
A-2	19	24.0	123
A-3	16	22.8	102
B-1	19	28.1	123
B-2	19	28.2	123
B-3	23	30.4	151

*Subline A or B obtained from an embryo.

In the age-dependent polyploidization of chick cells, the ploidy classes of 2C, 4C, 8C, 16C, 32C, 64C and 128C being defined as 2^nC, have been observed (Kaji and Matsuo, 1981). The time-course of polyploid nucleus accumulation in type A or B sublines is illustrated in Fig. 2. Among type A or B sublines, the age-dependent features of polyploid nucleus accumulation also were identical. As we have observed previously (Kaji and Matsuo, 1981), 4C nuclei in chick cells increased gradually after primary culture and 8C and more than 8C nuclei grew rapidly at the latest stage of cultures. However, the age-dependent feature of 4C nuclei in type A cells is somewhat different from that in type B cells. In type A cells, 4C nuclei increased almost linearly with advancing *in vitro* aging (Fig. 2a), but in type B cells, they grew progressively to reach to nearly 25% of the total nuclei around 50% lifespan completed (14 population doubling level), remained unchanged from 50 to 85% lifespan completed (14 to 24 population doubling level), and then increased towards 35% at the end of their lifespan (Fig. 2b).

The extent of age-related polyploidization is quantitatively expressed by polyploidization index (PI) (Kaji and Matsuo, 1981). Figure 3 shows the variations in the PI of type A and B cells as a function of lifespan completed. These PI curves are distinguishable from one another. These results show that among the sublines obtained from an embryo, the aging parameters of population doublings, population doubling rate and polyploidization are identical, and that the parameters of type A cells are inconsistent with those of type B cells. In addition, the time-course of multinucleation in type A cells has been found to be different from that in type B cells (Kaji and Matsuo, 1978*b*). These support the supposition that the difference of aging patterns between type A and B cells is not due to any artefact in culture, but due to their intrinsic nature.

Vol.
16
1981

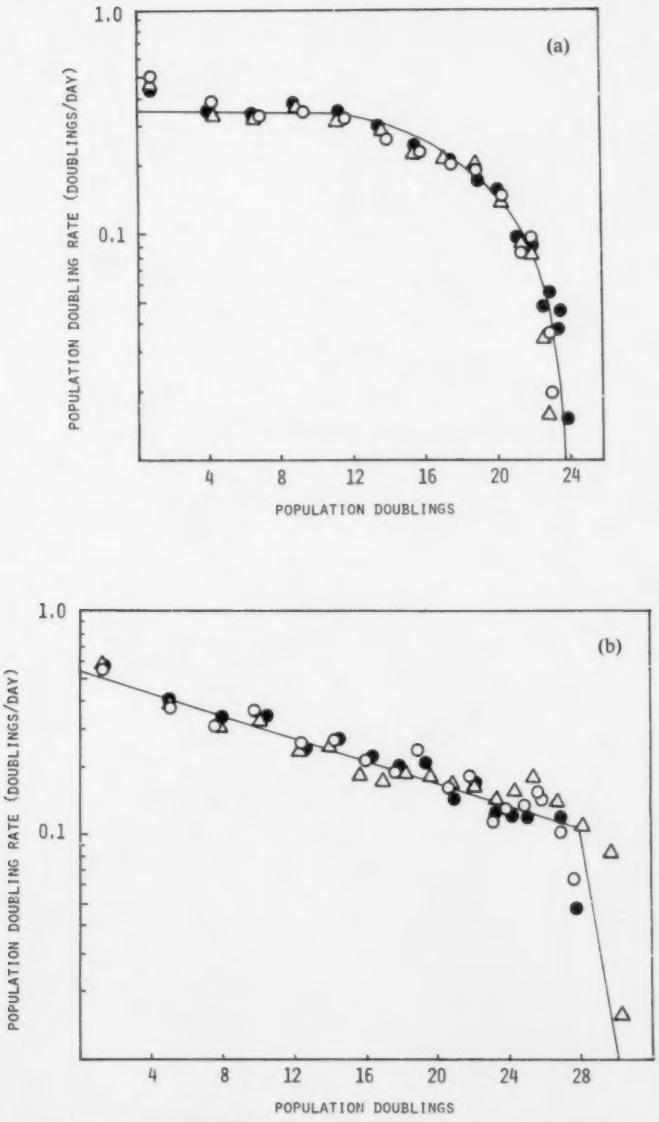

FIG. 1. The variations in population doubling rate during the lifespan of chick cells; (a) subline A-1 (—○—), A-2 (— ● —) and A-3 (—Δ—), (b) subline B-1 (—○—), B-2 (— ● —) and B-3 (—Δ—).

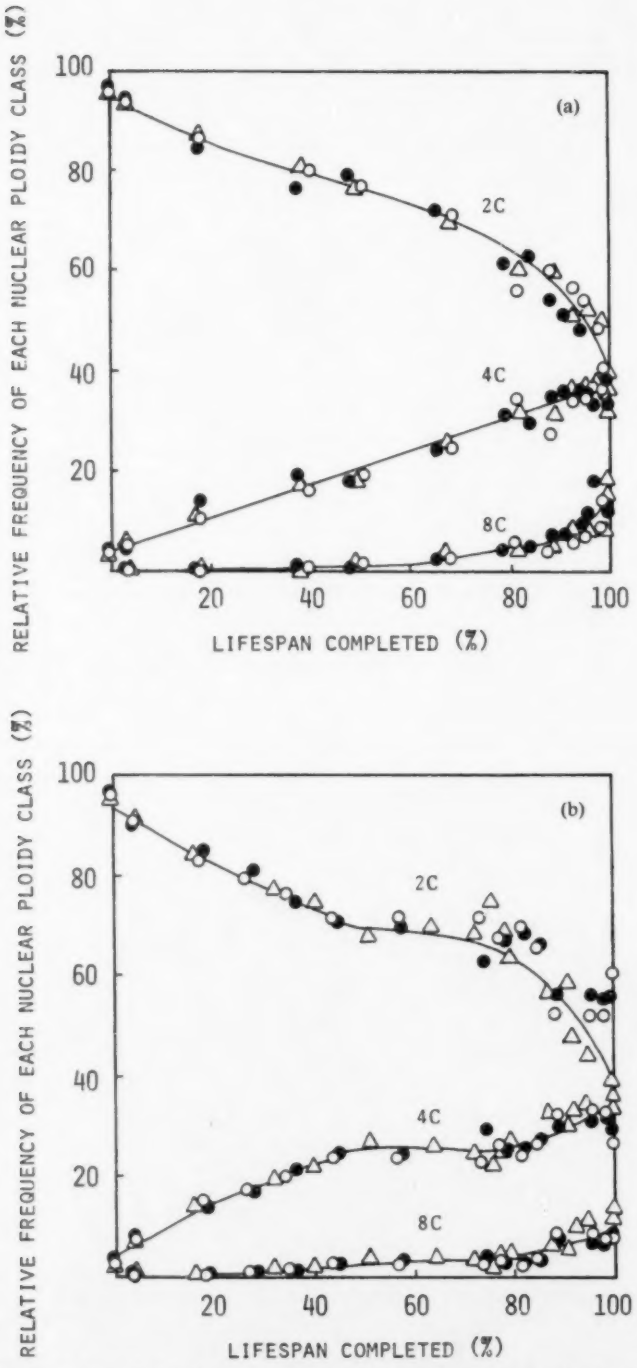

FIG. 2. The variations in the relative frequency of each nuclear ploidy class during the lifespan of chick cells; (a) subline A-1 (—○—), A-2 (— ● —) and A-3 (—Δ—), (b) subline B-1 (—○—), B-2 (— ● —) and B-3 (—Δ—). The percentages of more than 8C nuclei are not shown.

Vol.
16
1981

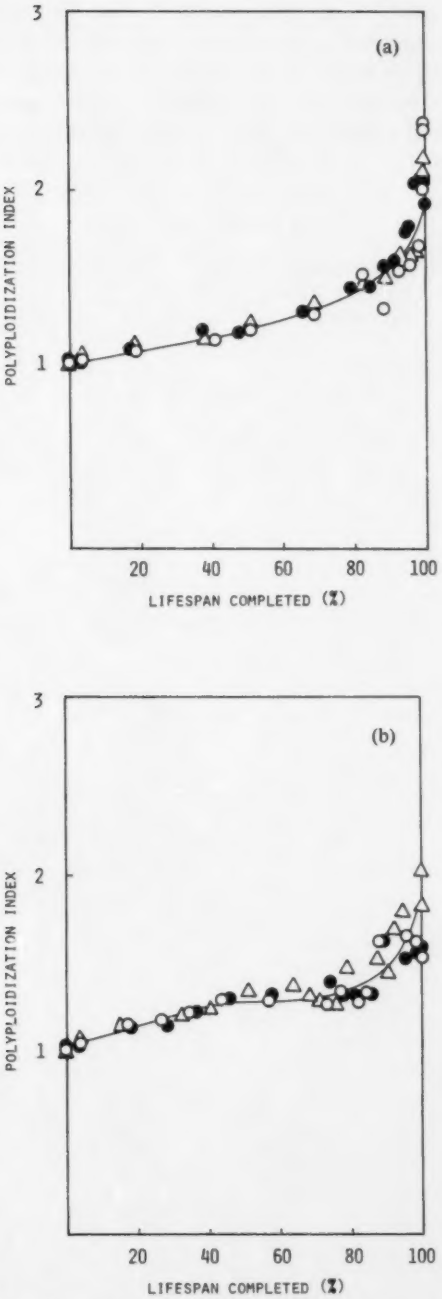

FIG. 3. The variations in polyploidization index during the lifespan of chick cells; (a) subline A-1 (—○—), A-2 (— ● —) and A-3 (—Δ—), (b) subline B-1 (—○—), B-2 (— ● —) and B-3 (—Δ—).

SUMMARY

Among the sublines of chick fibroblasts obtained from an embryo, *in vitro* lifespan was almost identical and age-related features of population doubling rate and polyploidization were quite similar. On the other hand, there were marked differences in these aging parameters between the populations of chick cells from different embryos. Presumably, the different aging patterns of chick cells observed are not due to any artefact in culture, but due to their intrinsic nature.

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AGE-RELATED CHANGES IN ENDOGENOUS RESPIRATION, RESPIRATORY CONTROL RATIO AND MALONATE INHIBITION OF OXYGEN CONSUMPTION OF LIVER HOMOGENATE OF MALE GARDEN LIZARDS

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Vol.
16
1981

INTRODUCTION

OUR PREVIOUS studies (Kara and Patnaik, 1980*a*) revealed that the respiratory rate of liver homogenate with added substrate declined with advancing age of male garden lizard. The mitochondrial marker enzyme SDH showed a peak activity in the lizards of the middle-aged group and then declined. Subsequent studies (Kara and Patnaik, 1980*b*) revealed that the thermal inactivation of SDH at 51 ± 1°C resulted in a higher degree of inhibition in middle-aged and old than in young lizards. The oxaloacetate inhibition of SDH activity and that of oxygen uptake of liver homogenate showed a similar pattern of age dependency. With increase in time of incubation the young lizards showed a partial recovery from inhibition of oxygen consumption but not the old.

These studies suggested that the functional integrity of mitochondria in male garden lizard liver is possibly deranged during ageing, resulting in eventual decline in respiratory metabolism. The present study provides additional evidence in favour of altered respiratory metabolism in aged lizards.

MATERIALS AND METHODS

Male garden lizards of the species *Calotes versicolor* (Daudin) collected from Berhampur localities during February to April were grouped as young, middle-aged and old (Kara and Patnaik, 1980*a*). Young lizards weighed between 5 and 16 g with a S-V length of 71–90 mm, middle-aged lizards had a body weight range of 20–39 g and a S-V length range of 93–114 mm whereas the corresponding values for old-age group were 45–70 g and 115–127 mm respectively. The approximate age of the lizards belonging to three different age-groups as confirmed by bone histology (Patnaik, unpublished data) were 0^+ (below 1 year), 1 and 2–4 years respectively. The endogenous respiration and the respiratory control ratio studies were done in lizards collected during February/March whereas the malonate inhibition studies were confined to the collection period of March/April. Lizards were maintained in the laboratory as described earlier (Kara and Patnaik, 1980*a*).

The oxygen uptake of liver homogenate prepared as described earlier (Kara and Patnaik, 1980*a*) was measured manometrically in a refrigerated Warburg respirometer (Ultra, Calcutta) using Ringer (Kara and Patnaik, 1980*a*) solution of pH 7.4 as the medium either without any added substrate (endogenous) or with exogenously added substrates (Na-succinate, 5 mM and Na-succinate, 5 mM + ADP, 0.3 mM). Malonate (Sodium malonate, 10 mM) inhibition of O_2 consumption was studied with Na-succinate (10 mM) as the substrate. The gas phase was air, and the duration of equilibration period 10 min. Readings were taken at 15 min intervals for 1 h. All the measurements were done at 33°C as it has been suggested (Bellairs, 1969) that the accritic temperature for many diurnal lizards is between 33° and 37°C.

The protein content of the tissue homogenate was measured colormetrically in a Spekol (Carl Zeiss) at 750 nm following the method of Lowry *et al.* (1951). The respiratory control ratio was calculated as

$$\frac{\mu lO_2 \text{ consumed (with Succinate + ADP/g wet wt or mg protein/h}}{\mu lO_2 \text{ consumed (with Succinate)/g wet wt or mg protein/h}}$$

RESULTS AND DISCUSSION

Chance and Williams (1956) used the term state I respiration (endogenous respiration) for respiration without any added substrate, state IV respiration (resting respiration) for respiration with high substrate and low ADP concentrations and state III (active respiration) for respiration with high substrate and high ADP concentrations for expressing respiratory capacity of mitochondria. These terms, though not exactly applicable to studies with homogenate, nevertheless can be used in a comparative sense to assess the changes in respiratory activity of mitochondria, as Strehler (1977) pointed out that studies with homgenate reflect a gross index of changes in mitochondria.

The endogenous respiration of liver declined between the young and middle-aged group remaining almost constant thereafter (Table 1).

TABLE 1. AGE-CHANGES IN VARIOUS PARAMETERS OF RESPIRATORY METABOLISM OF THE LIVER OF MALE GARDEN LIZARD

Age-group	$\mu l\ O_2$/g liver wet weight/h: Endogenous	p	With succinate	p	With succinate + ADP	ADP stimulation of oxygen consumption	Respiratory control ratio	% inhibition of oxygen consumption with succinate by malonate
Young (Y)	275.225 ±27.446 (8)	<0.001	1678.982 ±94.437 (7)	N.S.	1715.458 ±127.712 (9)	2.1	1.13±0.05 (8)	82.7±1.9 (8)
p	<0.02		<0.001				<0.1	N.S.
Middle-aged (M)	181.437 ±18.339 (7)	<0.001	1041.137 ±84.019 (8)	<0.01	1401.58 ±82.773 (7)	34.6	1.28±0.04 (9)	85.1±1.1 (7)
p	N.S.		N.S.				<0.01	N.S.
Old (O)	192.28 ±22.484 (5)	<0.001	1060.58 ±16.053 (5)	<0.01	1183.76 ±33.561 (5)	11.6	1.08±0.02 (7)	82.2±2.3 (8)
p (between Y and O)	<0.1		<0.001				N.S.	N.S.

Values are average ± SEM; number in parentheses indicate animal numbers, p values represented vertically show comparison between state I and state IV or between state IV and state III respiration, whereas p values represented horizontally show comparison between subsequent age-groups; N.S. is not significant at < 0.1 confidence level.

In endogenous respiration (state I) the supplies of both substrate and ADP are rate limiting. The fact that the liver homogenate of middle-aged and old lizards show a lower rate of respiration suggests that such a rate limitation is more evident in older lizards. In general at all ages the respiration with added substrate succinate was significantly higher than that of endogenous respiration. The state IV respiration also declined significantly with advancing age. ADP stimulation of respiration was marginal in liver homogenate of younger lizards, highest in middle-aged group and thereafter declined significantly in older lizards. Thus state III respiration also was significantly lower in the liver homogenate of older lizards.

The respiratory control ratio is the rate of respiration in presence of ADP to the rate in its absence. This measures the tightness of coupling during oxidative phosphorylation. The ratio showed a marginal rise in lizards of middle-aged group and declined significantly thereafter.

A survey of literature shows that such studies with tissue homogenate or mitochondria have shown conflicting results in ageing mammalian liver. Thus Barrows *et al* (1958) did

not find any age difference in the rates of endogenous oxygen uptake. Similarly Knook and Benzooijen (1973) indicated that in the parenchymal cells of rat liver there was no change in endogenous respiration and respiratory control ratio. Neither Gold *et al.* (1968) nor Wilson *et al.* (1975) found a significant age change in the respiratory control ratio of rat liver. On the other hand Chen *et al.* (1972) and Siliprandi *et al.* (1973) observed that the respiratory rate, ADP : O and respiratory control ratio of liver mitochondria declined significantly in ageing rats. Our results in garden lizards almost conform to the pattern observed by Chen *et al.* (1972) and Siliprandi *et al.* (1973) in rat liver supporting the view that the deranged respiratory metabolism in aged animals might be due to loss of functional integrity of mitochondria.

Malonate is a competitive inhibitor of the mitochondrial enzyme succinic dehydrogenase. We found earlier (Kara and Patnaik, 1980*a*) that the inhibition of enzyme activity of liver by malonate was significantly lower in older than in younger lizards, which suggested a greater stability of enzyme in the former thus deviating from the predictions of Orgel (1963). However, the present results with oxygen consumption of liver homogenate did not show a significant age-related alteration in the rate of inhibition raising doubt on the interpretation held earlier.

Vol.
16
1981

SUMMARY

The endogenous respiration and the respiration with added substrate (succinate) of liver homogenate declined significantly with advancing age of the male garden lizard, the changes between young and middle-aged being more prominent. ADP stimulation of respiration and thus the respiratory control ratio reached the peak level in middle-aged thereafter declining significantly in old. Malonate inhibition of oxygen uptake by liver homogenate with succinate as substrate was not age-dependent

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EFFECT OF AGE AND DIETARY COMPOSITION ON AORTIC COLLAGEN TO ELASTIN RATIO AND ON LIPID METABOLISM IN F-344 RAT

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Vol.
16
1981

INTRODUCTION

AGING is an important factor in bringing about qualitative changes in aortic collagen and elastin (Bartoš *et al.*, 1978; Fischer, 1976) and some dietary components, especially fat, raise the level of neutral lipids such as cholesterol and triglyceride in animal tissues (Kritchevsky, 1979; Takeuchi *et al.*, 1978).

In general, serum cholesterol, β- and pre-β-lipoprotein cholesterol levels tend to rise, and the serum α-lipoprotein cholesterol level tends to fall with aging and in aortic tissue of aged individuals, the total amount of collagen tends to increase and elastin to decrease (Kritchevsky, 1979). In aging experimental animals, the aortic collagen : elastin ratio (C/E) rises and the flexibility of the aorta decreases (Sheridan *et al.*, 1979; Bartoš *et al.*, 1978; Fischer *et al.*, 1977; Fischer, 1976; Fischer *et al.*, 1966).

Takeuchi *et al.* (1978) reported that in rats, lipidperoxide increases with age, and suggested some relationships between this increase and the elevation of serum lipid. As lipidperoxide may initiate or contribute to atherogenesis by acting as an inflammatory agent, the increase in the C/E ratio may relate to an elevation of the serum and hepatic lipidperoxide levels.

We studied the relationship between aortic C/E and the amount of lipidperoxide and lipid in the serum and liver of rats which had received diets of varying fat, cholesterol and cholic acid content for different periods.

MATERIALS AND METHODS

Animals and diets

Male Fisher-344 rats, 4 weeks of age, were housed 3 or 4 per wire cage, in a temperature- and humidity-controlled room with 12-hr cycles of light and darkness. They were checked daily and weighed weekly; fresh diet was provided twice a week.

The rats were divided into 4 groups; group I served as the control and received the basal diet; the other 3 groups received the experimental diet (Table 1) for 5, 11, 16 or 24 weeks. The respective diet and tap water were provided *ad libitum*.

For experimental determinations, the rats were fasted for 4 h (10:00–14:00 hrs), anesthetized with sodium pentobarbital and blood was withdrawn from the abdominal aorta, using a heparinized syringe. The liver and thoracic aorta were then removed. All samples were prepared within 1 h of anesthesia administration.

Lipid analyses

Serum cholesterol was determined by the cholesterol C-Test (Wako Pure Chemical Industries, Japan), triglyceride by the triglyceride B-test (Wako) and phospholipid by the phospholipid B-test (Wako). Hepatic cholesterol and triglyceride were extracted from homogenized liver tissue with a chloroform–methanol mixture (2 : 1, v/v), determination was by the above methods. Serum-free α-tocopherol content was determined by the method of Abe *et al.* (1975). Lipidperoxide in serum and liver was determined by the method of Yagi (1978) with tetraethoxypropane as the standard.

TABLE 1. COMPOSITION OF THE DIETS (%)

	Group I	II	III	IV
Sucrose	30.0	30.0	30.0	30.0
Wheat starch	33.5	33.4	33.4	32.9
Cellulose	0.5	0.5	0.5	0.5
Soybean oil	10.0	10.0	2.5	2.5
Hydrated coconut oil	0	0	7.5	7.5
Cholesterol	0	0.1	0.1	0.5
Cholic acid	0	0	0	0.1
Milk casein	20.0	20.0	20.0	20.0
Salt mixture*	4.0	4.0	4.0	4.0
Vitamin mixture†	2.0	2.0	2.0	2.0

*Phillips and Hart modified salt mixture IV. 100 g of the salt mixture contained: dipotassium phosphate, 32.2; calcium carbonate, 30.0; sodium chloride, 16.7; magnesium sulfate (hydrate), 10.2; calcium phosphate (dihydrate), 7.5; ferric citrate, 2.75; manganese sulfate, 0.51; potassium iodide, 0.08; copper sulfate, 0.03; zinc chloride, 0.025; cobalt chloride, 0.005.

†Vitamin mixture. 100 g of the vitamin mixture contained: vitamin A palmitate, 50,000 I.U.; calciferol, 4,000 I.U.; thiamine nitrate, 20 mg; riboflavin, 30 mg; nicotinamide, 200 mg; pyridoxine hydrochloride, 26 mg; folic acid, 10 mg; calcium pantothenate, 250 mg; cyanochobalamine, 20 μg; ascorbis acid, 750 mg; inositol, 1000 mg; choline chloride, 20,000 mg; α-tocopherol, 20 mg.

Aortic collagen and elastin analyses

Aortic collagen and elastin were separated by the autoclaving method (Fischer *et al.*, 1978; Fisher *et al.*, 1966), hydrolysis of collagen and elastin by hydroxyproline was carried out in 6N HCl for 18 h at 121°C under a pressure of 1.0–1.2 kg/cm^2. Hydroxyproline determination was performed by the method of Kivirikko *et al.* (1967).

RESULTS

Aging, dietary fat, cholesterol and cholic acid content had no effect on body weight gain. The liver weight decreased with age, but was increased in the rats receiving the cholesterol and cholic acid containing diet (Group IV).

The effect of aging, dietary fat, cholesterol and cholic acid content on the lipid level in serum and liver is shown in Table 2. In group IV, the serum cholesterol level was higher than in the other groups; dietary fat content had little effect on serum phospholipid or triglyceride levels. In all groups, the serum phospholipid and triglyceride levels increased with age, the same observation was made with respect to the cholesterol level, except in group IV rats. In this group, hepatic lipid content was markedly higher than in the other groups (Table 2).

The serum lipidperoxide level increased with age; in group IV, the hepatic lipidperoxide level was not markedly different from the other groups (Table 3). In group I, the serum lipidperoxide level was higher than in the other groups; in 9-week old rats of group I, the hepatic lipidperoxide level was higher than in the other groups. Age, dietary fat, cholesterol and cholic acid content had little effect on the serum α-tocopherol content.

The collagen content in the thoracic aorta tended to increase somewhat with age in all groups, while the elastin content tended to decrease (Table 4). The C/E ratio in the thoracic aorta increased markedly from weeks 9–15 in groups I and IV from weeks 15–20 in groups II and III.

TABLE 2. LIPID LEVELS IN RAT SERUM AND LIVER

Group	Age (weeks)	No. of rats	Serum (mg/dl) Cholesterol	Phospholipid	Triglyceride	Liver (mg/g) Cholesterol	Triglyceride
I	9	8	73.1 ± 5.80	206.1 ± 6.6	90.5 ± 29.4	5.91 ± 0.27	13.37 ± 2.83
	15	8	80.5 ± 5.30	167.9 ± 13.0	140.3 ± 34.4	4.85 ± 0.45	21.60 ± 2.50
	20	12	90.3 ± 10.6	211.6 ± 14.6	276.7 ± 45.2	5.03 ± 0.40	20.80 ± 2.70
II	9	8	62.2 ± 4.90	141.3 ± 14.3	102.1 ± 32.1	7.60 ± 1.17	25.10 ± 6.90
	15	12	85.0 ± 13.4	182.6 ± 23.0	191.9 ± 64.5	10.34 ± 1.10	33.90 ± 5.70
	20	12	130.3 ± 11.2	251.3 ± 16.0	317.2 ± 42.6	6.80 ± 0.70	26.30 ± 3.80
	30	12	133.3 ± 17.0	261.9 ± 29.4	411.6 ± 112.3	8.14 ± 1.01	39.80 ± 7.90
III	9	8	82.8 ± 5.20	192.1 ± 12.2	189.9 ± 26.9	7.61 ± 0.88	22.50 ± 4.20
	15	12	112.5 ± 9.00	237.7 ± 17.7	268.1 ± 39.5	6.85 ± 0.49	22.0 ± 6.90
	20	12	131.5 ± 10.4	250.0 ± 16.6	361.4 ± 42.9	6.20 ± 1.20	29.30 ± 3.20
IV	9	8	168.1 ± 15.2	183.8 ± 9.5	118.4 ± 15.4	22.61 ± 2.65	44.60 ± 7.40
	15	12	132.6 ± 11.5	258.2 ± 14.6	221.6 ± 30.8	14.62 ± 2.27	36.80 ± 4.10
	20	12	133.3 ± 18.9	226.8 ± 27.1	274.4 ± 72.6	16.80 ± 1.60	49.80 ± 4.80
	30	12	189.9 ± 31.8	269.3 ± 39.0	274.7 ± 99.9	12.60 ± 2.78	64.50 ± 23.3

Each value represents mean ± SD.

Vol. 16 1981

TABLE 3. SERUM AND HEPATIC LIPIDPEROXIDE AND SERUM α-TOCOPHEROL CONTENT IN RATS

Group	Age (weeks)	No. of rats	Serum Lipidperoxide* (n moles/ml)	Serum α-tocopherol (mg/dl)	Liver Lipidperoxide* (n moles/g)
I	9	8	7.23 ± 0.54	0.273 ± 0.019	239.4 ± 32.7
	15	9	8.88 ± 1.11	0.301 ± 0.030	279.5 ± 21.2
	20	12	12.26 ± 1 67	0.280 ± 0.023	184.9 ± 17.9
II	9	8	3.61 ± 0.47	0.179 ± 0.042	118.8 ± 26.4
	15	12	4.26 ± 0.63	0.241 ± 0.022	249.5 ± 24.3
	20	12	7.16 ± 0.52	0.221 ± 0.029	328.8 ± 84.2
	30	11	10.55 ± 2.35	0.455 ± 0.091	326.2 ± 67.8
III	9	8	2.97 ± 0.24	0.179 ± 0.021	149.5 ± 11.4
	15	12	4.11 ± 0.62	0.279 ± 0.047	230.5 ± 21.7
	20	11	7.08 ± 1.07	0.166 ± 0.022	239.1 ± 66.8
IV	9	8	3.73 ± 0.24	0.246 ± 0.022	100.8 ± 14.0
	15	12	4.92 ± 0.48	0.173 ± 0.010	81.3 ± 13.2
	20	11	9.28 ± 0.67	0.233 ± 0.029	254.3 ± 49.4
	30	12	7.50 ± 1.19	0.310 ± 0.069	248.1 ± 38.1

Each value represents mean ± SD.
*Estimated as malondialdehyde values.

DISCUSSION

The present study showed that the dietary content of soybean and/or hydrated coconut oil did not affect the serum cholesterol, phospholipid or triglyceride content in rats. The serum lipid level increased with age, a finding which coincides with that of Kritchevsky

(1979). The presence of cholesterol and cholic acid in the diet increased the serum cholesterol level (Table 2), suggesting that these dietary constituents affected the lipoprotein metabolism and cholesterol synthesis in the liver and intestines, and that hepatic deposition of cholesterol and triglyceride occurred (Bochenek and Rodgers, 1979; Nakabou *et al.*, 1978; Krumdieck and Kong-Jey Ho, 1977; Takase *et al.*, 1977).

Takeuchi *et al.* (1978) have shown that in rats, the serum lipidperoxide level increases with age and decreases with the feeding of a cholesterol-containing diet. In our study, in the experimental groups, the serum lipidperoxide level increased by approximately 43% between the 15th and 20th week of life (Table 3). In groups II and III, the serum cholesterol and lipidperoxide levels increased linearly between weeks 9 and 20 ($r = 0.975$ and $r = 0.853$, respectively).

TABLE 4. COLLAGEN AND ELASTIN CONTENT IN THE THORACIC AORTA OF RATS

Group	Age (weeks)	No. of rats	Collagen	Percent Elastin	C/E
I	9	8	6.90 ± 0.56	17.65 ± 0.69	39.07 ± 3.49
	15	9	8.18 ± 0.50	16.15 ± 0.93	50.80 ± 3.80
	20	12	8.41 ± 0.46	16.16 ± 1.10	52.24 ± 4.66
II	9	8	9.06 ± 0.80	23.57 ± 1.41	38.74 ± 3.60
	15	12	8.43 ± 0.75	19.30 ± 1.23	43.95 ± 2.89
	20	12	10.29 ± 0.59	16.40 ± 0.92	63.40 ± 3.67
	30	11	10.19 ± 0.42	17.85 ± 1.09	57.24 ± 2.95
III	9	8	7.04 ± 0.29	17.88 ± 1.79	39.84 ± 4.83
	15	12	9.60 ± 1.73	24.25 ± 2.27	40.12 ± 8.72
	20	12	9.99 ± 0.60	14.98 ± 1.07	67.14 ± 7.17
IV	9	8	7.89 ± 1.05	17.79 ± 0.92	43.58 ± 1.43
	15	12	7.69 ± 0.53	15.07 ± 1.19	51.22 ± 3.61
	20	12	7.63 ± 0.47	12.94 ± 1.60	59.69 ± 6.69
	30	12	9.79 ± 0.69	17.67 ± 1.12	55.57 ± 4.54

Percent values were calculated on the basis of the wet weight of the thoracic aortas. C/E; Collagen : elastin ratio, calculated for each aorta. The values are mean ± SD.

The C/E ratio in the thoracic aorta markedly increased between weeks 15 and 20 in rats of groups II and III (Table 4). The serum lipidperoxide level of 9 and 15 week old group I rats (control) was twice as high as in the experimental groups; in the controls, there was a marked increase in the C/E ratio of the thoracic aorta between weeks 9 and 15. These observations indicate that the increase in serum lipidperoxide is correlated with an increase in the C/E aortic ratio.

The elastin content has been reported to decrease with age, while the collagen content does not (Bartoš and Ledvina, 1978; Fischer, 1976). We found that in rats receiving the cholesterol-supplemented diet, the age-related increase in the C/E aortic ratio was delayed; dietary fat content did not manifest this effect. The period during which the serum cholesterol, phospholipid and triglyceride levels became elevated, corresponds with the period in which the aortic C/E ratio and the serum lipidperoxide level increased.

The relationship between atherosclerosis and lipidperoxide has been studied (Wilson, 1976). Our findings lead us to suggest that changes in the structure of the rat thoracic aorta are induced by lipidperoxide at an early stage of aging.

In rats which became spontaneously hypertensive at the age of 9–20 weeks, this period corresponded with an increase in both the C/E ratio of the thoracic aorta and the serum lipidperoxide (Newman and Langer, 1978; Okamoto *et al.*, 1972). Ooshima *et al.* (1975, 1974) reported that the decrease in aortic collagen and the increase in collagen synthesis in hypertensive rats are related with an increase in aortic lysyloxidase activity and Sheridan *et al.* (1979) have suggested that the aortic collagen content increases in hypertensive rats.

Future emphasis should be directed at studying the relationship between the lipid-peroxide level, and the structure and synthesis of collagen and elastin in aortic tissue during the aging process.

SUMMARY

The effects of aging, dietary fat, cholesterol and cholic acid content on the aortic collagen : elastin ratio and on the lipid level in rat serum and liver were investigated. Body weight gain was not affected by the dietary fat, cholesterol or cholic acid content; feeding of a cholesterol and cholic acid containing diet increased the liver weight, especially in younger rats. With age, serum cholesterol, triglyceride, phospholipid, and lipidperoxide levels increased, while the hepatic cholesterol and triglyceride content did not. Supplementary dietary cholesterol and cholic acid increased the lipid level in serum and liver. Quantitative and qualitative changes in the thoracic aorta began at an early stage of aging, they were correlated with an increase in serum lipidperoxide, cholesterol and triglyceride.

Vol. 16 1981

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AGE RELATED DECREASE IN NUCLEAR AND NUCLEOLAR SIZE IN HYPODERMAL CELLS OF THE ROTIFER

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Abstract—With advancing age, there is a continuous and significant decrease in the diameter of both nucleus and nucleolus in hypodermal cells of the rotifer, *Asplanchna brightwelli*. The decrease in nucleolar diameter is most pronounced (63%) and may be related to a decrease in synthetic activity of these cells. The nucleus decreases in diameter by 25% during the 6 day lifespan of the rotifer. Decrease in nuclear size may be characteristic of ageing both in long-lived and in short-lived post-mitotic cells.

Vol.
16
1981

INTRODUCTION

THE PURPOSE of this study was to determine whether quantitative changes take place in nuclear or nucleolar size during ageing in the short-lived rotifer *Asplanchna brightwelli*. This species lends itself well to ageing studies because of its short life span of 5–6 days, and because it can easily be maintained in the laboratory (Enesco and Holtzman, 1980). Since the hypodermal cells of the rotifer presented the most uniform appearance and are most clearly visible in squash preparations, they were selcted for cytological measurements. These cells are involved with the formation of the cuticle, and are thus located within the transparent coat. In the adult rotifer, the hypodermal cells form a syncytium (Brodie, 1970).

A decrease in nuclear size takes place during ageing in mammalian neurons, and has been described in cerebellar granule cells of rabbits (Cammermeyer, 1963) and in the cerebral cortex of rats (Brizzee *et al.*, 1975). As for nucleoli, they are reported to be paler, vacuolated or even missing in neurons of older animals (Brizzee *et al.*, 1975).

We were interested to determine whether the cytological changes characteristic of ageing in mammalian post-mitotic cells would also appear during ageing of post-mitotic cells in a short-lived invertebrate, the rotifer.

MATERIALS AND PROCEDURES

Culture methods and determination of lifespan

Cultures of the rotifer *Asplanchna brightwelli* clone 4B61 were obtained from Dr. John Gilbert, Dartmouth College, Hanover, NH. They were cultured on a cerophyll medium, inoculated with *Escherichia coli* and *Paramecium caudatum*. So that the lifespan and age of individual rotifers could be accurately determined, rotifers of known birth date were maintained singly in the wells of Costar No. 3524 Tissue Culture Chambers. These sterile 24-well dishes were obtained from Costar, Cambridge, MA, and held approx. 2.5 ml of medium. The preparation of the medium, the maintenance of the stock cultures, the handling of individual rotifers of known age, and the determination of lifespan for rotifers are described in an earlier paper (Enesco and Verdone-Smith, 1980).

Cytological studies

To prepare squash preparations, rotifers of known ages were first mouth-pipetted to a new culture well containing cerophyll medium, free of any living organisms. From this medium, an individual rotifer was transferred to a pre-cleaned microscope slide with two drops of medium. It was then covered gently with a coverslip. The rotifers usually remained relatively intact in the squash preparation.

Measurements of nucleolar and nuclear diameter were made under phase contrast at 40× magnification on a Zeiss Ultraphot microscope. Measurements were made quickly to avoid desiccation, using a calibrated ocular micrometer. These measurements were carried out on rotifers ranging from 1–6 days of age with 7–15 rotifers per age group.

RESULTS

Lifespan

The average lifespan of a population of 122 individual rotifers was 4.43 days. This is in accordance with a previous report from our laboratory which placed the average lifespan for *Asplanchna brightwelli* at 4.54 days (Enesco and Holtzman, 1980). Maximum longevity was 6 days.

Nuclear and nucleolar diameter

The sizes of both the nuclei and nucleoli of the hypodermis were determined in rotifers of increasing age. Table 1 presents the average diameter and volume of nuclei and nucleoli of the hypodermis in groups of rotifers ranging in age from 1 to 6 days. The diameter of the nucleus decreased from 11.20 to 8.30 μm during the lifespan of the rotifer (a 25% decrease), while the diameter of the nucleolus decreased from 6.05 to 2.25 μm with age (a 63% decrease). This decrease in size of both nucleus and nucleolus is illustrated in Fig. 1, which shows hypodermal nuclei from 2-day, 4-day and 6-day-old rotifers. The volume of both nucleus and nucleolus was calculated at all ages, and these values are also presented on Table 1. The decrease in volume with age is much more pronounced for the nucleolus than for the nucleus.

TABLE 1. CHANGES IN AVERAGE DIAMETER AND VOLUME OF THE HYPODERMAL NUCLEUS AND NUCLEOLUS WITH AGE. THE NUCLEOLAR/NUCLEAR RATIOS ARE INCLUDED (Nu/N)

Age (days)	*N*	Diameter (μm) Nucleolus	Nucleus	Nu/N	Volume (10^{-6} mm^3) Nucleolus	Nucleus	Nu/N
1	7	6.05	11.20	0.540	0.116	0.736	0.158
2	15	5.00	10.45	0.480	0.065	0.598	0.110
3	15	4.00	9.65	0.415	0.034	0.471	0.071
4	13	2.80	8.90	0.315	0.011	0.369	0.031
5	12	2.20	7.80	0.282	0.006	0.248	0.022
6	10	2.25	8.30	0.271	0.006	0.299	0.021

Calculation of a nucleolar to nuclear ratio was carried out for each age group. If the diameter or volume of the nucleus were decreasing proportionately with age, one would expect this ratio to remain constant. Since the data in Table 1 show that the ratio is getting smaller with age, we see that the nucleolus is decreasing in size at a much greater rate than is the nucleus. Statistical analysis based on the correlation coefficient for the slope of the line confirmed that the nucleolar to nuclear ratio decreases significantly with age ($p > 99.5$).

DISCUSSION

Our results show that the size of both the nucleus and the nucleolus steadily decrease with age in hypodermal cells of the short-lived rotifer *Asplanchna brightwelli.* It is

Vol.
16
1981

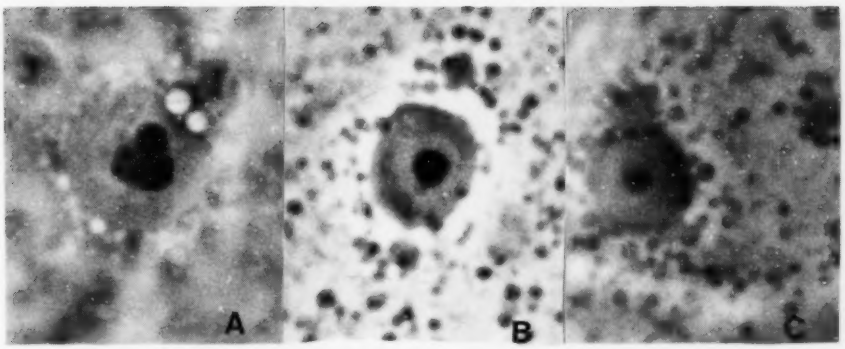

FIG. 1. Nuclei and nucleoli of rotifer hypodermal cells decrease in size with age. A, 2 days; B, 4 days; and C, 6 days of age.

interesting that we observe the same trend in post-mitotic cells of this short-lived invertebrate as that observed in post-mitotic cells of longer-lived higher organisms.

In interpreting our results we should keep in mind that a decrease in size of either nucleus or nucleolus may be correlated with a decline in function. Both nuclei and nucleoli increase in size when cells are stimulated to maximal synthetic activity, such as during regeneration of the liver or regeneration of the *Acetabularia* rhizoid, or during an increase of activity in glandular cells. This size increase is reviewed by Grundmann (1966) and by Gabe and Arvy (1961). Since nuclei and nucleoli of actively synthesizing cells are often larger than those of less active cells, we must ask whether our observations are correlated not only with age, but with cell activity.

Fortunately, there have been a number of studies on the hypodermal cells of the rotifer in relation to cuticle formation to which we can refer for information on cell function. The hypodermal cells of the rotifer are responsible for the formation of an extracellular cuticle as described by Koehler (1965, 1966) and by Brodie (1970). The cuticle is a glycoprotein secretion product formed by the hypodermal cells shortly before birth. According to Brodie (1970) cuticle formation takes place only in the embryo and newborn animal. Adult rotifers are incapable of regenerating a cuticle. Our observations show that the nucleolus is particularly large in the young rotifers, and decreases rapidly in size as the rotifer ages. Since the nucleolus and ribosomes must be involved with glycoprotein synthesis in the hypodermal cells of the newborn rotifers, our observations of rapidly declining nucleolar size correlate well with declining glycoprotein synthesis.

The nucleus decreases less dramatically than the nucleolus in our studies (25% decrease in diameter as compared to 63% for the nucleolus). The gradual decrease in size of the nucleus is thus more likely to represent age-specific cytological change.

It is of interest that a decrease in nuclear size with age is observed both in long-lived and very short-lived species.

Vol. 16 1981

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ALLOXAN-INDUCED DIABETES IN YOUNG VS OLD SPRAGUE–DAWLEY RATS

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Vol. 16 1981

INTRODUCTION

THERE is a progressive deterioration of glucose metabolism in animals and in man concomitant with progressively increasing hyperinsulinemia associated with the aging process (Andres, 1971; Davidson, 1979; Reaven *et al.*, 1979). Blumenthal (1978) suggested that diabetes is a prototype of aging. Further, diabetes may serve as a prototype of accelerated aging or progeria, e.g. Cushing's or Werner's syndrome, as expressed by premature atherosclerosis, osteoporosis, senile cataract formation (Wexler, 1976), and more fundamentally, vascular basement membrane thickening and reduction of the replicative lifespan of cultured fibroblasts (Goldstein and Hanley, 1979).

The increasing incidence of diabetes, particularly maturity-onset diabetes, cardiovascular disease, and the ever-expanding gerontological population prompted an attempt to simulate this clinical problem experimentally and to investigate the possibility that experimental diabetes might serve as a prototype of accelerated aging. Young and old, male and female, Sprague–Dawley rats were compared concerning those spontaneous metabolic alterations which occur with aging, e.g. hyperglycemia, hyperlipidemia, and increased blood urea nitrogen (BUN). In addition, young and old male and female rats were compared according to their ability to withstand the pathophysiological changes which attend alloxan-induced diabetes.

METHODS AND PROCEDURE

Previous experience with Sprague–Dawley rats made severely diabetic with alloxan necessitated the provision of a large number of animals initially to allow for the great number of deaths anticipated during the first 7 days of alloxan-induced ketosis and unrelenting diabetes. Adult male and female virgin rats 6–7 months old, raised in our Animal Colony, were classified as "young", male and female virgin rats, 18–20 months of age, also raised in our Animal Colony and successfully kept free of respiratory and overt disease, were classified as "old". The young and old rats were raised under the same conditions of diet, lighting, temperature, and humidity control.

All of the experimental animals were subjected to an 18-h fast prior to the injection of alloxan. Four groups of male and female, young and old rats (n = 24/group) were set aside to serve as non-treated baseline controls. A single, subcutaneous injection of 10 mg of alloxan (Eastman Kodak) per 100 g of body weight was given to both the young and old rats. Insulin therapy was withheld deliberately. Throughout the course of the experiment, all of the animals were fed a regular commercial rat chow (Purina) which was relatively low in fat (4%).

The systolic blood pressure of each animal was recorded by means of the Freedman–Freed indirect microphonic manometer and tail cuff apparatus. At autopsy, each of the animals was anesthetized lightly with Seconal and blood withdrawn by heparinized syringe from the abdominal aorta. The blood was spun in a refrigerated centrifuge and the serum was frozen and stored until time of analysis. Automated techniques (Auto-analyzer: Technicon) were used to measure triglycerides, free fatty acids, total cholesterol, glucose and BUN. In addition, serum corticosterone, the main adrenocortical steroid produced by the rat, was also measured by an automated fluorometric method (Guillemin *et al.*, 1959). The heart and main arteries of each animal were examined for any evidence of vascular disease. Pertinent organs from each animal were trimmed and weighed for purposes of gravimetric analyses and then fixed in 10% buffered, neutral formalin for histopathologic examination. In addition to hematoxylin and eosin, special stains were used to demonstrate metachromasia

and mucopolysaccharides (Alcian and toluidine blue, Hale stain), elastic tissue (Verhoeff–van Gieson), calcium (von Kossa), beta-cell granularity of the islets of Langerhans (aldehyde fuchsin–Ponceau), and lipids in frozen sections (Sudan black B). The gravimetric and biochemical data were subjected to statistical analyses using analysis of variance and Student's *t*-test prescribed by Snedecor and Cochran (1967). Values of p greater than 0.05 were considered to be not significant.

RESULTS

General observations

Within 2 h of the single injection of alloxan, all of the animals manifested copious quantities of glucose in their urine. Within 24 h, all of the animals were hyperglycemic, hyperlipidemic, and had severe ketosis. The classical signs of overt diabetes, i.e. polyphagia, polydypsia, and polyuria, were also present. Mortality was high, reaching a critical zenith on the 4th day post-alloxan—those animals which succumbed at this time manifested a severely fatty liver at autopsy. Forty-six per cent of the young animals succumbed during the first 4 days of alloxan-induced diabetes vs 77% of alloxan-treated old rats. This acute high mortality rate subsided in both young and old rats, and although the initially high circulating lipid and glucose levels also gradually subsided, the polyuria, polydypsia, polyphagia, hyperglycemia and hyperlipidemia persisted. Testing with Labstix® demonstrated considerably more urinary protein and glucose excretion in the older rats. Because of progressively worsening diabetes and deaths, particularly in the old rats, the experiment was terminated 60 days post-alloxan.

Gravimetric changes

Young male and female rats became overtly obese as a result of diabetes, whereas the old rats became emaciated (Table 1). By gross observation, all of the diabetic animals manifested enlarged, hemorrhagic adrenal glands but only the young male and female rats exhibited statistically significant increases in adrenal weight compared to controls (Table 1). The thymi of all diabetic animals were significantly ($p < 0.001$) involuted (Table 1). The hearts and kidneys of the young male and female diabetic rats were significantly ($p < 0.001$) heavier than their untreated partners, whereas the hearts and kidneys of the old male and female diabetic rats weighed significiantly ($p < 0.001$) less than controls (Table 1). The testes of young and old diabetic males were atrophic and although the ovaries of young and old diabetic rats were involuted, these differences were not statistically significant (Table 1). The above observations obtained whether organ or body weights were expressed on an absolute weight basis or as ratio of 100 × organ weight : body weight.

Blood pressure

The systolic blood pressure was significantly ($p < 0.001$) elevated in the young male and female diabetic rats; blood pressure levels were lowered in the old rats (Table 1).

Blood chemistry

The male and female untreated old rats exhibited significantly ($p < 0.001$) greater hyperglycemia than their younger partners (Fig. 1). Circulating glucose levels were supernormal in the young diabetic rats (Fig. 1). The older diabetic rats were hyperglycemic compared to their non-treated partners but the severity of their hyperglycemia was considerably less than the young diabetic rats (Fig. 1). The older control rats manifested significantly ($p < 0.001$) higher blood triglyceride and total cholesterol levels than the

Vol. 16 1981

TABLE 1. ORGAN WEIGHTS, BODY WEIGHT, AND BLOOD PRESSURE OF YOUNG VS OLD, MALE VS FEMALE RATS WITH AND WITHOUT ALLOXAN-INDUCED DIABETES

		No. of rats	Final body wt (g)	Adrenal	Thymus	Heart	Kidney	Testes/ ovary	Blood pressure (mmHg)
				(mg)					
Male	Young 6–7 months								
	Control—No RX	24	356 ± 12	19 ± 4	308 ± 10	1018 ± 19	1088 ± 20	1902 ± 14	92 ± 2
	Alloxan diabetes	13	398 ± 10*	26 ± 1*	105 ± 11**	1298 ± 24**	1768 ± 43**	1785 ± 16**	124 ± 2**
	Old 18–20 months								
	Control—No RX	18	459 ± 24	43 ± 5	75 ± 14	2169 ± 22	2227 ± 87	1896 ± 88	142 ± 4
	Alloxan diabetes	11	393 ± 13**	48 ± 4	34 ± 11**	1573 ± 31**	1715 ± 46**	1714 ± 62**	132 ± 2*
Female	Young 6–7 months								
	Control—No RX	24	245 ± 10	23 ± 2	235 ± 10	810 ± 13	795 ± 22	41 ± 3	82 ± 2
	Alloxan diabetes	12	265 ± 4*	34 ± 2**	88 ± 10**	991 ± 31**	1169 ± 35**	31 ± 5	117 ± 3**
	Old 18–20 months								
	Control—No RX	20	369 ± 9	45 ± 2	108 ± 7	1048 ± 19	990 ± 25	52 ± 4	138 ± 3
	Alloxan diabetes	11	279 ± 6**	48 ± 2	75 ± 11**	989 ± 31**	827 ± 43**	43 ± 5	129 ± 2**

Note: All values cited are the mean ± standard error.
*$p < 0.05$, alloxan-diabetic vs controls.
**$p < 0.001$, alloxan-diabetic vs controls.

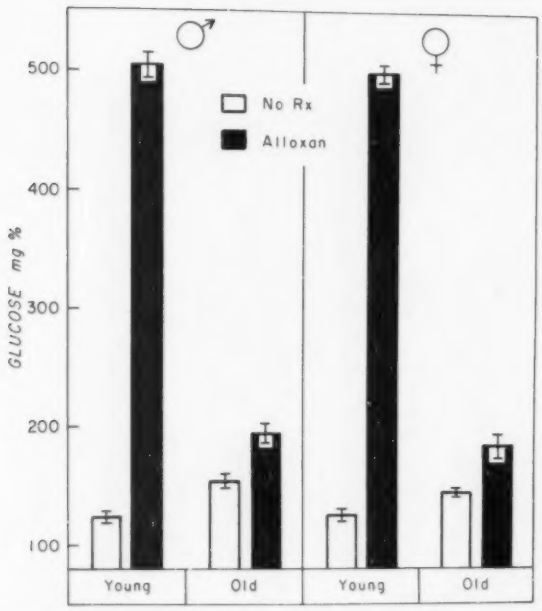

FIG. 1. Barogram depicting the differences in circulating glucose levels between young (7–9 months) vs old (20–22 months) male vs female Sprague–Dawley rats receiving no treatment or after having been made severely diabetic with alloxan. All of the animals were autopsied 60 days after the experimental animals had been made diabetic. The height of each column depicts the mean ± standard error; young male controls, $n = 24$, diabetic, $n = 13$; old male controls, $n = 18$, diabetic, $n = 11$; young female controls, $n = 24$, diabetic, $n = 12$; old female controls, $n = 20$, diabetic, $n = 11$. This same protocol follows for Figs. 2–6.

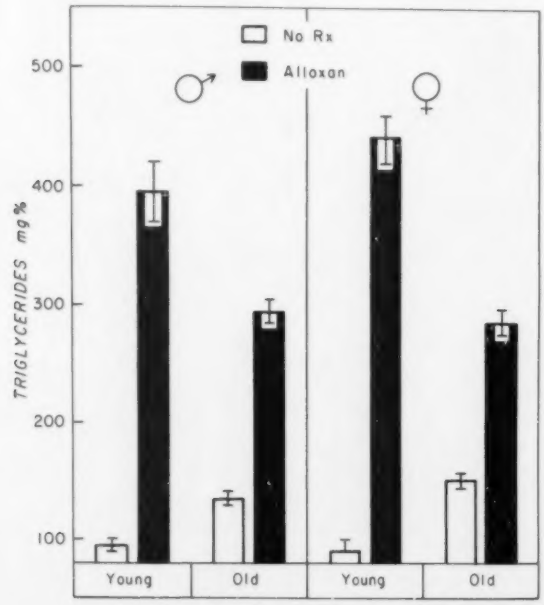

FIG. 2. Changes in circulating triglyceride levels.

young control rats but aging appeared to be associated with reduced levels of circulating free fatty acids ($p < 0.001$, Fig. 3) Paradoxically, although both the young and old rats displayed grossly-visible hepatic lipidosis and hyperlipidemia, the free fatty acid levels were signficantly ($p < 0.001$) below normal in the old diabetic rats, whereas all of the remaining young and old diabetic rats manifested severely elevated triglyceridemia (Fig. 2), free fatty acids (Fig. 3), and total cholesterol (Fig. 4). As in the case of blood glucose (cf. Fig. 1), the triglyceride levels of the older diabetic rats were not as severely elevated as in the young diabetic rats (Fig. 2). The older untreated rats showed increased BUN levels (Fig. 5). The increased BUN levels associated with aging were greatly exacerbated by the induction of diabetes exceeding, in most cases, the elevated BUN levels induced by severe diabetes in the younger animals (Fig. 5). Despite considerably heavier adrenal glands and involuted thymi in the older control animals (Table 1), only the old male rats secreted more corticosterone ($p < 0.05$) than their younger confreres. Older females secreted slightly higher (not statistically significant) amounts of corticosterone compared to young females (Fig. 6). Despite the greatly enlarged and hemorrhagic adrenal glands (and severely involuted thymi (Table 1)) encountered in diabetic animals, the blood corticosterone levels were significantly ($p < 0.001$) elevated only in the young diabetic animals; older diabetic animals manifested little or no differences in circulating corticosterone levels compared to their untreated brothers and sisters (Fig. 6).

Vol. 16 1981

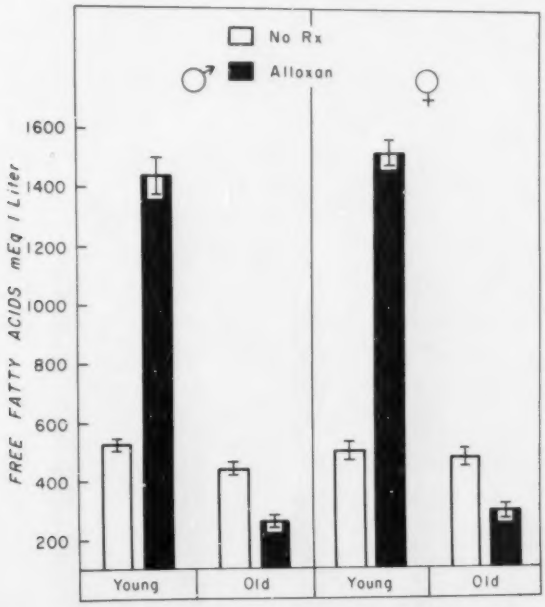

FIG. 3. Changes in circulating free fatty acid levels.

Gross and microscopic pathology

Thirty-two per cent of old untreated, male and female rats displayed scattered foci of myocardial fibrosis, whereas 67% of the old male and female diabetic rats displayed grossly-visible foci of fibrosis. None of the young rats showed any untoward alterations

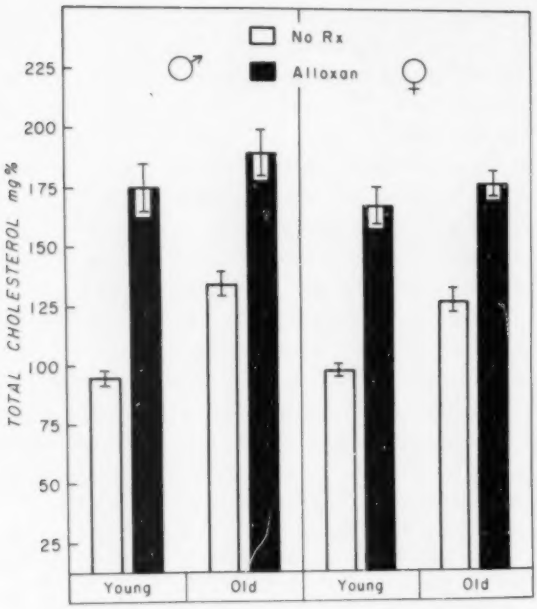

FIG. 4. Changes in circulating total cholesterol levels.

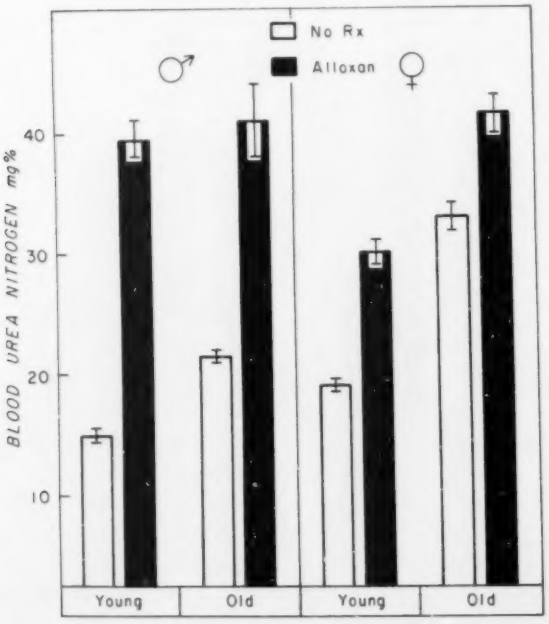

FIG. 5. Changes in circulating blood urea nitrogen levels.

in the heart. All of the diabetic animals manifested severe fatty livers and severe involution of the islets of Langerhans. The old control rats displayed a variable incidence of islet hyperplasia accompanied by varying degrees of beta cell degranulation. The adrenal cortices of the young diabetic rats were hyperplastic and extensively depleted of lipid, whereas the adrenal glands of the older diabetic rats were greatly enlarged, hemorrhagic, with foci of thromboses and microcortical infarcts, hyperplasia of the zona glomerulosa, and extensive lipid depletion from the fascicular and reticular zones. Despite greatly elevated BUN levels (Fig. 5) in diabetic old rats, the kidneys of these animals displayed relatively mild degenerative changes, i.e. glomerular hyperplasia with early glomerulosclerosis, tubular colloid, and beginning interstitial nephritis. The testes and ovaries of all diabetic animals showed advanced involution and atrophy.

Vol. 16 1981

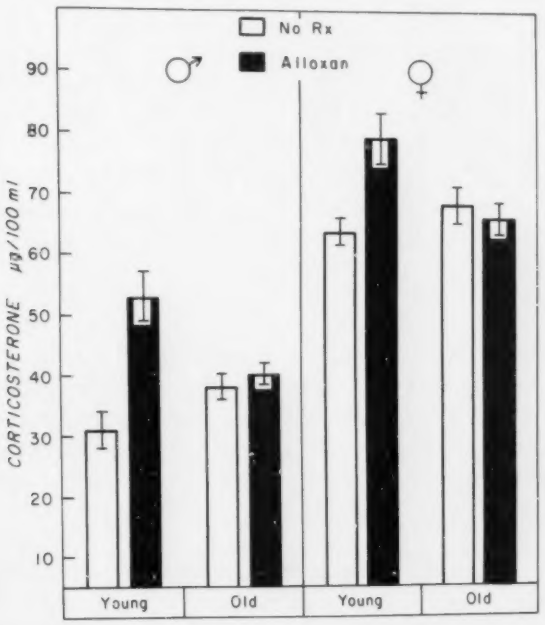

FIG. 6. Changes in circulating corticosterone levels.

The most outstanding histopathologic finding was the appearance of a 36% incidence of early arteriosclerotic lesions concentrated primarily in the arch of the aorta and proximal portions of the carotid arteries in old diabetic rats. These lesions consisted of raised intimal plaques composed of mixed fibrocellular proliferate, edema, and mucoproteinaceous material which stained strongly positive for mucopolysaccharide (glycosaminoglycans), and densely populated by intensely basophilic mesenchymal cells which became rounded and appeared to be consistent with beginning cartilaginous metaplasia. Despite fatty infiltration of the liver and hyperlipidemia (Figs. 2–4), these arterial lesions contained no lipid. Instead, there were intimal spicules of calcification surrounded by actively differentiating, intensely basophilic mesenchymal cells (Fig. 7).

DISCUSSION

This investigation demonstrates that naturally-occurring diabetes does accelerate the aging process and advanced, alloxan-induced diabetes greatly exacerbated the process of accelerated aging, e.g. the statistically significant greater incidence and severity of arterial lesions in alloxan-diabetic old rats than in old control rats. That is, we have found that virgin Sprague–Dawley rats do not develop arteriosclerosis spontaneously until they are senile (Wexler, 1979). Repeatedly bred male and female rats develop obesity, diabetes, and arteriosclerosis spontaneously when they are 6–9 months old and are considered prematurely old (Wexler, 1964). In this experiment, the 20–22 month old control (virgin) rats did not exhibit any evidence of arterial disease, whereas 36% of old diabetic rats manifested early arteriosclerotic lesions morphologically identical to those found in senile virgin rats (Wexler, 1979). This would underscore the conclusion that diabetes, particularly severe diabetes, will cause acceleration of the aging process.

Particularly provocative is the dichotomous anabolic-catabolic response of diabetic young vs old rats, i.e. diabetes-induced obesity in young vs emaciation in old rats. Since there is a well-established relationship between obesity and hypertension, there could be a correlation between the diabetes-induced high blood pressure in young rats and the lowered blood pressure in the emaciated diabetic old rats. Similarly, despite the appearance of severe hepatic lipidosis of apparently equal severity in young and old diabetic rats; old rats, with and without diabetes, could not mobilize glucose and lipids, especially free fatty acids, as effectively as their younger partners. There is a growing consensus that the hyperlipidemia of diabetes or other metabolic dyscrasias is not only related to lipid synthesis but to lipid mobilization, transport, and disposal. It is also provocative that although the old control rats manifested progressively worsening hyperglycemia and hyperlipidemia associated with the aging process (Andres, 1971; Davidson, 1979; Reaven *et al.*, 1979; Lacko and Davis, 1979; Masoro *et al.*, 1979), the old rats did not mobilize glucose or lipids to the same extent as the young diabetic rats under the duress of overt diabetes. This would suggest that old rats lose their capacity to respond to the challenge of severe diabetes. It is enigmatic how this inability to mobilize glucose and lipid in old diabetic rats is related to their accelerated arteriosclerosis, albeit that the arterial lesions were free of lipid.

The inability of the older rats to respond to the metabolic stress of severe diabetes may be due to failure of their aging pituitary–adrenal axis. There is conflicting opinion whether pituitary–adrenal function increases, remains unchanged, or becomes hypofunctional with age (Hess and Riegle, 1970; Landfield *et al.*, 1978; Tang and Phillips, 1978; Wexler, 1979). The greatly enlarged and hemorrhagic adrenal glands concomitant with the severe thymus gland involution in the older diabetic rats attest to a period, prior to the time of autopsy, during which pituitary gland secretion of ACTH and adrenocortical secretion must have been of intense degree. The decreased adrenocortical steroidogenesis in these diabetic old rats is in keeping with the inability of old diabetic rats to mobilize glucose and lipid, and is in conformity with the suggested breakdown of the hypothalamic–pituitary–adrenal responsiveness to severe diabetes. In ancillary studies, it has been shown that during the acute stage (4–7 days) of alloxan-induced diabetes, there is an acute and considerable rise in circulating corticosterone levels in Sprague–Dawley rats (Wexler, 1970; Wexler *et al.*, 1970; Wexler, 1971). With persisting diabetes (4–8 weeks), corticosterone levels recede to subnormal levels. This would indicate an initial hyperadrenocorticism in response to the stress of diabetes, followed by a period of "adrenal exhaustion" or

Vol.
16
1981

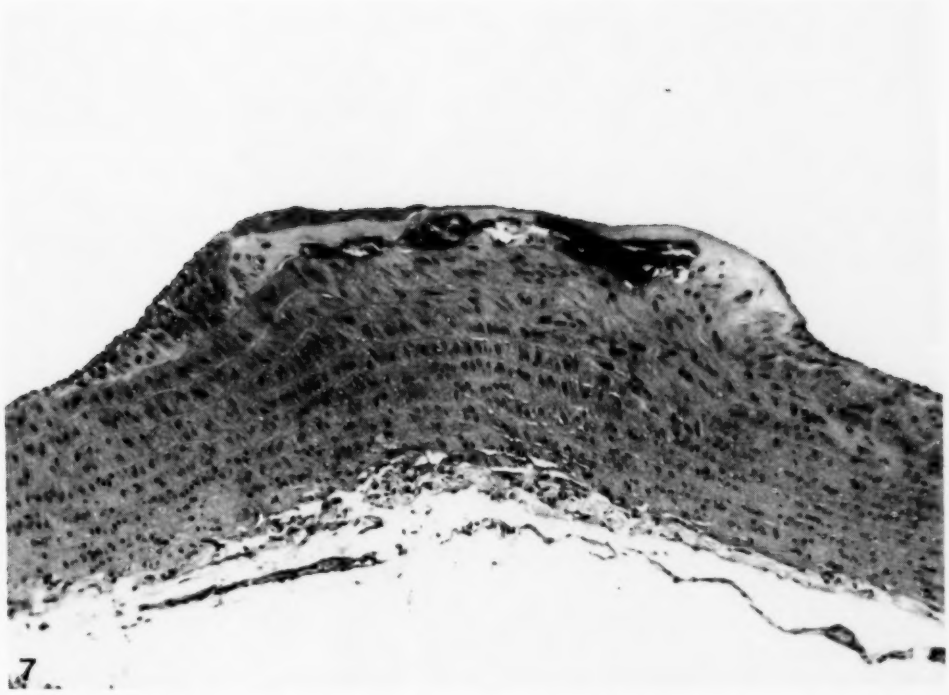

FIG. 7. Aorta of a 20 month old, male, virgin, Sprague–Dawley rat after 60 days of severe alloxan diabetes, showing an intimal lesion which is identical to the arterial lesions which appear spontaneously in senile virgin rats or in repeatedly bred rats of a younger age. The raised intimal plaque contains edema and a mucoproteinaceous material (light grey in photo) which stains strongly positive for mucopolysaccharide. The fibrocellular proliferation on the shoulder (left side) of the intimal plaque is composed of mesenchymal cells which differentiate into large intensely basophilic rounded cartilaginous cells (see isogenous group of cells on right side of plaque) which are responsible for the intimal calcific deposit (black material in photo). H + E, × 80.

hypoadrenocorticism. It is suggested that the fundamental defect in the older diabetic rats resides within the hypothalamus and pituitary gland. The concentration of ACTH is normal in the pituitary glands of old and alloxan-diabetic rats (Devecerski and Frawley, 1963; Wexler *et al.*, 1970; Kraus, 1973). Although the pituitary glands of such animals are capable of synthesizing and releasing ACTH under resting conditions, there appears to be a definite block in hypothalamic–pituitary function with impaired release of preformed ACTH (Devercerski and Frawley, 1963; Wexler *et al.*, 1970; Kraus, 1973). The consensus is that during aging the pituitary and adrenal glands retain their capability to synthesize and secrete ACTH and corticoids. It is an age-associated defect at the hypothalamic–pituitary level which makes older animals unable to respond properly to stress (Hess and Riegle, 1970; Tang and Phillips, 1978; Wexler, 1976). The inability of the old diabetic rats to mobilize glucose and lipid, their reduced blood pressure, and their inability to secrete corticosterone could be due to a block in hypothalamic–pituitary ACTH release rather than to some aging-induced intrinsic defect in adrenal steroidogenesis. Similarly, the testicular and ovarian atrophy found in these animals could be due to a defect in hypothalamic–pituitary gonadotrophic hormone release.

Vol.
16
1981

SUMMARY

This investigation shows that old male and female, Sprague–Dawley rats are unable to cope as effectively as young rats with the metabolic stress of alloxan-induced diabetes. Although the aging process is associated with progressively increasing hyperglycemia, hyperlipidemia, hypertension, and an increased proclivity toward arteriosclerosis, the pathophysiologic response to acutely-induced diabetes is not the same in young vs old rats. Old rats were found to be unable to mobilize glucose and lipids, or to secrete corticosterone as effectively as young rats. Concomitantly, although the young rats remained resistant to the pathogenesis or arterial diesease, the older diabetic rats manifested definite acceleration of naturally-occuring arterial disease relegated to senile Sprague–Dawley rats. It is suggested that the premature appearance of arterial disease in these diabetic rats may be equated with premature aging. It is also suggested that the spectrum of unusual pathophysiologic changes observed in the older rats was due to an acute phase of hyperadrenocorticism in response to the stress of acute diabetes followed by a period of adrenocortical hypofunction and that alterations in hypothalamic–pituitary–adrenal function may condition animals toward premature aging.

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THE EFFECT OF HAMSTER AGE ON U.V.-INDUCED UNSCHEDULED DNA SYNTHESIS IN FRESHLY ISOLATED LUNG AND KIDNEY CELLS

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Vol. 16 1981

INTRODUCTION

CELLULAR systems, including human tissues and cultured cells, have a number of enzymatic repair pathways to remove DNA damage induced by endogenous and exogenous chemical and physical agents (Hanawalt *et al.*, 1979; Grossman *et al.*, 1975; Lindahl, 1979). One repair mechanism, excision repair, involves enzymatic recognition of the damage, then excision of the lesion and of a number of adjacent nucleotides, repair synthesis in the excised region using the undamaged complementary strand as template, and finally ligation. Excision repair acts upon DNA lesions induced by ultra-violet (u.v.) radiation and various chemical mutagens and carcinogens (Regan and Setlow, 1974; McCaw *et al.*, 1978; Kleihues and Margison, 1974; Goth and Rajewsky, 1974). Deficiency of this mechanism is associated with enhanced mutagenesis (Witkin, 1966; Maher *et al.*, 1976) and carcinogenesis (Goth and Rajewksy, 1974; Robbins *et al.*, 1974). In addition, the level of excision repair, as measured by u.v.-induced unscheduled DNA synthesis (UDS) in isolated fibroblasts correlates with the life span of several mammalian species (Hart and Setlow, 1974; Hart *et al.*,1979; Paffenholz, 1978), implying that accumulation of DNA lesions is a significant aspect of aging and that DNA repair promotes longevity.

Little (1976) has suggested that an age-associated decline in the capacity of cells to perform DNA repair could contribute to normal aging. Such a decline could result either from a genetically programmed reduction in repair capability during development or a gradual deterioration of genetic information including that for repair enzymes.

In the present study we have investigated these possibilities by studying the DNA repair capacity in different tissues of genetically identical hamsters over much of the life span of the animals. Ultra-violet-induced UDS was measured in lung and kidney cells (this paper), and liver and brain cells (Gensler, 1981) isolated from inbred hamsters of various ages. Ultra-violet-induced unscheduled DNA synthesis was chosen for determination of repair capacity because this is the repair measurement which correlates with maximum life span (Hart and Setlow, 1974), u.v. dose to DNA can be readily controlled compared with chemical treatment and repair synthesis is a late step in the excision repair pathway. The golden hamster was used as the tissue source because its maximum life span of 765 days (Kirkman and Yau, 1972) is convenient for studying aging effects and was reported by Hart and Setlow (1974) to have a two-fold greater level of u.v.-induced UDS than either rat or mouse. Cell suspensions were prepared from fresh tissue and used rapidly in order to study viable post-mitotic neurons and to maintain as much of the normal *in vivo* regulation of DNA repair as possible. It has been found that after several transfers *in vitro,* excision repair is reduced in rodents (Peleg *et al.*, 1977; Chan and

Walker, 1977). We observed that the levels of excision repair in lung and kidney are maintained, with some fluctuation, from youth through at least 2/3 of the maximum life span of the animal. Similar evidence for a lack of decline of repair over a similar portion of the life span in brain and liver cells also is presented in the companion paper (Gensler, 1981). In addition, we observe an early increase in the number of lung and kidney cells performing u.v.-induced excision repair, suggestive of a developmental turn on.

MATERIALS AND METHODS

Tissue preparation

Inbred LHC/Lak Syrian golden hamsters were originally obtained from Charles River Lakeview, and additional hamsters were raised locally. Hamsters were killed with ether at various ages. Lungs and kidneys were removed and placed in petri dishes which were kept on ice. The tissues were minced at a ratio of 1 g of tissue to 10 ml of Hank's Balanced Salt Soluation (HBSS, Baltimore Biological Laboratory). Cells were then adjusted to a concentration of 5×10^6 cells/ml in HBSS.

Irradiation

Each cell suspension (4 ml) was placed in a plastic petri dish and exposed at a distance of 40 cm to 30 s of u.v. radiation from a 15-W General Electric germicidal lamp (output primarily at 254 nm) at a dose rate of 100 Jm^{-2} min^{-1}. The depth of the cell suspension was approx. 1 mm and the suspension was shaken during the period of irradiation. Steps of the experiment which were performed after u.v. irradiation and before removal of medium containing tritiated thymidine (^{3}HTdR) were carried out in a darkened room to prevent photoreactivation. Unirradiated and exposed samples were centrifuged at 730 ***g*** for 10 min. The supernatants were then discarded, and the cells were incubated in 3.2 ml of Dulbecco Modified Eagle's Medium (DMEM, Grand Island Biological Co.) containing 10% fetal calf serum (Grand Island Biological Co.), 10% horse serum (Grand Island Biological Co.), penicillin at 50 units ml^{-1}, streptomycin at 50 μg ml^{-1}, and 20 μCi ml^{-1} ^{3}HTdR (New England Nuclear, 51.4 Ci $mmole^{-1}$). After 2 h of incubation at 37°C, a drop of the cell suspension was mixed gently with a drop of 0.2% trypan blue solution. The per cent of cells excluding trypan blue was determined as a measure of viability. Only those cell samples in which viability exceeded 80% were included in the results. The cells were washed once with DMEM and pelleted by centrifugation at 730 ***g*** for 10 min.

Fixation of cells

Cells in the pellet were fixed in the centrifuge tube by layering alcohol : acetic acid (3 : 1) over them. After 5–15 min the fixative solution was removed and about 5–10 drops of 2% aceto-orcein solution was mixed with the cells. Two drops were placed on each slide and spread with a coverslip. After freezing the cells to the slide by placing the slide on dry ice, the coverslip was removed. The cells were then hydrated by passing the slides through 20% ethanol, 10% ethanol, and water, consecutively.

Radioautography

Slides were dipped into Kodak NTB-3 Emulsion at 42°C and exposed for 1 week. They were then developed in Kodak Developer D19 (3 min at 17°C) and fixed in Kodak General-purposer Fixer (3 min at 17°C). Nuclei undergoing replicative DNA synthesis are densely covered with grains, whereas nuclei participating in repair DNA synthesis have a relatively small number of grains which can be counted. An average of 1200 cells per irradiated sample and 1100 cells per unirradiated sample were examined for identifying and counting those cells undergoing unscheduled DNA synthesis, those in S-phase, and those undergoing no DNA synthesis. In addition grain counts were performed on about 200 of the irradiated cells in the sample. In the unirradiated cell samples, very few cells were lightly labeled. Therefore grain counts were made for as many as occurred in the total number of cells observed (as listed in Tables 1 and 2).

Inhibitor assay

To determine if an inhibitor of repair was released during tissue mincing, an equivalent weight of tissue was put through a fine stainless steel mesh into: (a) 4 ml or (b) 40 ml of HBSS. After centrifugation at 2000 ***g*** for 10 min, a supernatant solution or, as a control, plain HBSS was used to suspend lung cells at a concentration of 5×10^6 ml^{-1}. After 15 min at room temperature, 4 ml of these lung cell suspensions were exposed to u.v. radiation and incubated with ^{3}HTdR as described above. Radioautographs were prepared and grain counts over cells were performed. The percentage of cells exhibiting u.v.-induced UDS and the mean grain count of repairing lung cells were compared in the control and treated samples to determine if repair was inhibited by either supernatant.

RESULTS

Unscheduled DNA synthesis in irradiated lung cells isolated from hamsters of various ages

Unscheduled DNA synthesis (UDS), or non-S-phase DNA synthesis, is considered to represent the DNA synthesis step of excision repair, based mainly on its correlation with dimer excision and repair replication in rodent and human cells (Painter and Cleaver, 1969: Peleg *et al.*, 1977; Cleaver, 1968). Nuclei covered with 5–55 grains/cell were considered to be performing UDS. Nuclei with more than 55 grains were considered to be in S-phase. Most nuclei in S-phase had a distinctive appearance, being densely covered with grains.

Table 1 presents the data on unscheduled DNA synthesis for irradiated and unirradiated lung cells from hamsters of different ages. The mean grain count and standard error as well as the absolute number and per cent of cells undergoing UDS are given. Repair

TABLE 1. UNSCHEDULED DNA SYNTHESIS IN U.V.-IRRADIATED AND UNIRRADIATED LUNG CELLS ISOLATED FROM HAMSTERS OF VARIOUS AGES

		Cells with UDS						
U.V. radiation	Age (days)	Mean grain count*	Standard error	Number	Per cent of total cells	Repair synthesis†	Cells in S-phase (%)	Viability‡ (%)
+	4	9.0	0.81	19	2.1	19.2	2.0	96
−	4	9.3	0.87	9	0.8	7.8	3.3	94
+	5	8.5	0.31	216	14.9	126	6.3	
−	5	10.1	1.67	28	1.5	15.4	4.4	97
+	5	11.0	0.39	261	13.6	150	3.3	
−	5	10.8	1.08	61	1.9	20.8	4.1	94
+	6	16.9	0.73	165	35.1	595	6.2	
−	6	12.3	1.12	37	2.8	34.3	4.4	96
+	6	12.6	0.50	189	37.6	473	3.8	
−	6	9.6	0.76	47	3.5	33.6	4.1	99
+	8	15.8	0.44	321	37.7	595	4.7	98
−	8	10.1	2.95	8	1.1	11.1	2.9	
+	133	14.3	0.52	192	41.7	598	0.9	80
−	133	6.9	0.45	20	2.0	13.6	0.6	
+	147	13.2	0.65	158	27.5	361	2.2	98
−	147	7.4	0.69	12	1.8	13.0	2.5	
+	195	13.2	0.46	154	32.9	434	1.6	
−	195	6.3	0.75	4	1.0	6.1	2.0	93
+	309	9.3	0.88	32	27.2	252	1.0	
−	309	5.5	0.38	8	1.5	8.1	1.7	99
+	316	11.9	0.42	149	30.0	357	0.4	95
−	316	7.3	0.98	10	1.1	7.8	0.8	
+	320	9.5	0.27	191	34.6	328	0.5	99
−	320	8.3	1.23	16	2.2	18.2	0.6	
+	527	16.9	0.52	187	52.1	881	0.7	
−	527	8.3	0.70	25	2.2	19.4	0.4	96
+	534	10.9	0.37	162	25.0	275	0.6	92
−	534	8.7	1.39	19	2.8	24.6	1.0	
+	547	12.9	0.44	183	38.7	498	0.9	99
−	547	6.5	0.45	10	0.8	5.2	1.0	

*The mean grain count is for nuclei undergoing unscheduled DNA synthesis, i.e. displaying 5–55 grains.

†Repair synthesis is the product of the mean grain count times the per cent of total cells showing UDS.

‡Viability was initially measured in the unirradiated aliquot of cells, on the supposition that the irradiation would result in reduced viability. However, it was found that viability of the irradiated cells remained high during the incubation, so treated cells were assayed in subsequent experiments.

Vol. 16 1981

synthesis (defined below), per cent of total cells in S-phase, and per cent viability are also presented. We define "repair synthesis" as the product obtained by multiplying the per cent of cells undergoing repair by the mean grain count per repairing cell to give the mean grain count per 100 cells. As shown in Table 1, the per cent of total irradiated lung cells which exhibited UDS increased from 2–15% on days 4 and 5 to 35–38% by day 6. From day 6 to day 547 the proportion of irradiated lung cells performing repair fluctuated around a mean of 35%. During this age span, the mean number of unirradiated lung cells showing UDS was 1.9%. To test whether the low proportion of irradiated cells showing repair during the 4–5 day period was due to an inhibitor in the cell preparations, the inhibitor assay described in "Materials and Methods" was used. No repair inhibitor was found in the supernatant solutions obtained from mincing 4 day or adult lung tissue. Repair synthesis in irradiated lung cells increased 7-fold between days 4 and 5, and another 4-fold between days 5 and 6, then remained at this increased level, with some fluctuation, to day 547. The per cent of total cells in S-phase dropped in both irradiated and unirradiated cells as the hamsters reached adulthood.

Figures 1 and 2 depict the mean values obtained from Table 1 by grouping the data in clusters of three individual hamsters of similar ages. In Fig. 1(a) the per cent of lung cells undergoing UDS is plotted vs. the age of the donor hamster. The sharp increase in number of cells undergoing repair between days 5 and 6 is evident, as well as the maintenance of a high number of repairing cells throughout the age span studied. The mean grain count per repairing cell is shown in Fig. 1(b). The increase in mean grain count of irradiated over unirradiated lung cell after day 5 is evident. Figure 1(c) shows the decrease in number of lung cells in S-phase for both irradiated and unirradiated cells as the hamster reaches adulthood. Repair synthesis of lung cells is plotted in Fig. 2, in which the sharp rise between days 5 and 6 is seen, as well as the sustained high level of repair synthesis to at least day 547.

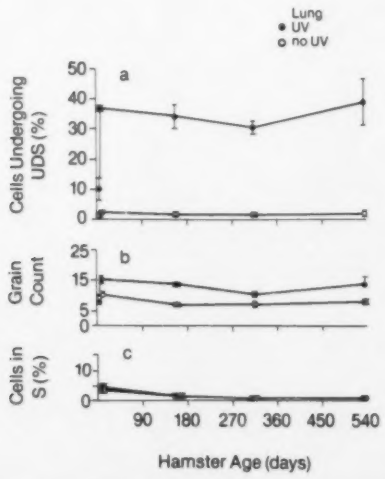

FIG. 1. The influence of hamster age on DNA synthesis in u.v.-irradiated and unirradiated lung cells. Mean values ± S.E. obtained with three individual hamsters of similar age are plotted. ●, Irradiated cells; ○, unirradiated cells. (a) Frequency of lung nuclei showing u.v.-induced UDS at different hamster ages. (b) The mean grain count of lung nuclei displaying UDS at different ages. (c) Frequency of lung nuclei in S-phase at different ages.

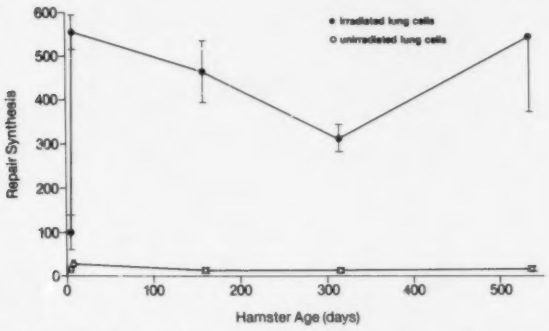

FIG. 2. Repair synthesis in u.v.-irradiated and unirradiated lung cells at different hamster ages. Mean levels ± S.E. of repair synthesis in cells of three individual hamsters of similar age are plotted. Repair synthesis is the mean grain count over 100 nuclei, obtained by multiplying the per cent of nuclei undergoing UDS by the mean grain count per repairing nucleus. ●, Irradiated lung nuclei; ○, unirradiated lung nuclei.

Vol. 16 1981

Unscheduled DNA synthesis in irradiated kidney cells isolated from hamsters of various ages

The per cent of total irradiated kidney cells undergoing UDS, presented in Table 2, rose from a mean of 7.5 on days 4–6 to a mean of 17.0 on day 9, and, except for one hamster, remained between 11.6 and 23.1 to day 527. The repair synthesis values of irradiated kidney cells rose from a mean of 83 on days 4–6 to a mean of 242 on day 9, and thereafter fluctuated between 135 and 465 (except for one value) to day 527. The low percentage of cells exhibiting UDS (1.5) on day 309 may be an experimental error, but with adequate viability and no objective criterion to discard the value, it was retained. The values for day 527 were included in Table 2 even though the cell viability at the end of the period of incubation with ^{3}HTdR was only 65% (i.e below the 80% viability usually required for acceptance) because there were limited numbers of such old hamsters and the values for this age were similar to the high values obtained in young adult hamsters, as if the cells were repairing during the incubation.

Figures 3 and 4 summarize the experiments with kidney cells. Each point represents the mean value of three or four individual hamsters of similar age. Figure 3(a) shows the 2-fold rise in the per cent of irradiated kidney cells which underwent repair between days 5 and 9, as well as the higher number of repairing cells in irradiated vs. unirradiated cells throughout the age span considered. The mean grain count of irradiated cells was slightly more than that of unirradiated cells on days 4–9; this difference increased sharply by day 55 and remained generally higher, although with fluctuation, thereafter (Fig. 3b). The per cent of kidney cells in S-phase declined as the hamster reached young adulthood and then remained low (Fig. 3c). Repair synthesis in irradiated kidney cells is seen in Fig. 4 to have increased 2-fold between days 5 and 9, and to have remained generally higher in irradiated cells than in unirradiated cells to day 527. No evidence was found for inhibition of u.v.-induced UDS in lung cells after exposure to supernatants from kidney minces at the concentrations described in "Materials and Methods".

DISCUSSION

Effect of hamster aging upon lung and kidney repair capacities

The amount of u.v.-induced unscheduled DNA synthesis in cells isolated from lung and kidney of hamsters did not show progressive decline from youth to over 520 days,

TABLE 2. UNSCHEDULED DNA SYNTHESIS IN U.V.-IRRADIATED AND UNIRRADIATED KIDNEY CELLS ISOLATED FROM HAMSTERS OF VARIOUS AGES

U.V. radiation	Age (days)	Cells with UDS: Mean grain count*	Standard error	Number	Per cent of total cells	Repair synthesis†	Cells in S-phase (%)	Viability (%)
+	4	8.8	0.48	80	7.3	65.6	3.9	87
−	4	7.6	0.89	8	1.4	13.3	4.2	
+	5	12.5	0.64	158	11.5	144	4.4	89
−	5	7.8	2.23	13	1.2	9.6	3.4	
+	6	10.6	1.11	70	3.7	39.2	1.3	
−	6	10.9	2.65	12	1.2	12.9	1.3	98
+	8	9.7	0.54	116	8.9	85.9	1.2	95
−	8	7.3	1.86	3	0.8	5.6	0.3	
+	9	17.6	1.75	52	19.1	335	2.6	90
−	9	6.9	0.99	9	2.2	15.0	2.6	
+	9	9.9	0.40	194	15.0	149	2.0	92
−	9	6.3	0.52	7	0.9	5.6	1.8	
+	55	22.9	1.13	137	13.1	300	0.2	
−	55	7.1	0.24	65	3.3	23.7	0	86
+	57	19.9	1.37	74	13.5	269	0	90
−	57	8.0	0.49	26	3.7	29.3	0	
+	83	21.8	1.24	112	11.8	256	0.2	90
−	83	9.9	1.08	14	1.4	13.9	0	
+	239	15.1	0.75	144	15.5	233	0.2	84
−	239	5.0	0	3	0.2	0.8	0.1	
+	239	20.2	0.82	180	23.1	465	0	86
−	239	9.3	1.13	15	1.1	9.9	0.1	
+	246	16.3	0.718	167	17.6	286	0.1	87
−	246	6.7	0.576	20	1.6	10.4	0.1	
+	309	9.2	1.27	20	1.5	14.1	0	
−	309	7.0	1.03	6	1.6	11.3	0.2	93
+	316	11.6	0.807	73	11.6	135	0.4	98
−	316	8.6	0.598	22	2.4	20.3	0	
+	320	9.6	0.278	186	20.4	195	0.2	95
−	320	8.3	1.23	16	2.6	21.4	0.2	
+	527	18.9	0.885	162	23.1	438	0.1	
−	527	9.6	0.663	22	0.1	0.8	0	65

*See legend to Table 1.
†See legend to Table 1.

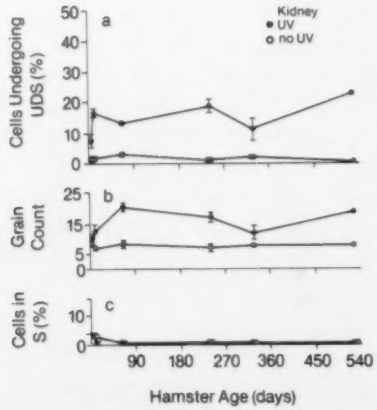

FIG. 3. DNA synthesis in u.v.-irradiated and unirradiated kidney cells at different hamster ages. The legend is similar to that of Fig. 1.

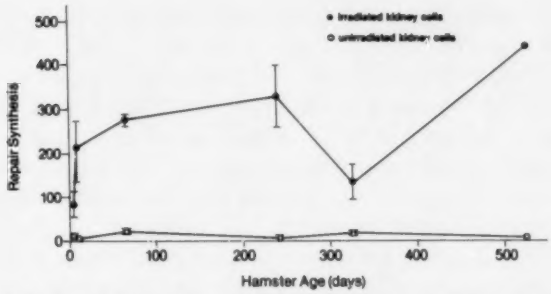

FIG. 4. Repair synthesis in u.v.-irradiated and unirradiated kidney cells at different hamster ages. The legend is similar to that of Fig. 2.

a period which comprises 2/3 of their maximum life span. Maintenance of young adult levels of repair was found in brain and liver also (see companion paper, Gensler, 1981). Absence of a systematic change in UDS suggests that the accumulation of genetic lesions observed by other investigators in similar tissues during this age period (Chetsanga *et al.*, 1975; Chetsanga *et al.*, 1977; Price *et al.*, 1971; Murthy *et al.*, 1976) is not due to decline in repair capacity as the host ages. Rather, DNA damage may accumulate due to the difference between the rate at which lesions occur and the rate at which the normal adult tissue repairs such damage.

Vol. 16 1981

Factors determining unscheduled DNA synthesis levels

The rate of incorporation of ^{3}HTdR during repair DNA synthesis may be determined by a number of factors, which have been listed and discussed by Hart and Setlow (1974) who used UDS as the basis for comparing repair capacity in the same tissue for different mammalian species: "(a) the number of photoproducts per unit u.v. fluence, (b) the amount of DNA per cell, (c) the specific activity of the internal thymidylate pools, (d) the average size of the repaired regions, and (e) the number of repaired sites per unit length of DNA". They also discussed evidence indicating that factor (e) was the most important parameter. Further information relating to these factors has become available.

(a) *The number of photoproducts per unit u.v. fluence.* The state of the chromatin structure may influence the efficiency with which u.v. fluence induces thymine dimers in mammalian cells. In Chinese hamster cells and HeLa cells, 30% and 50% more thymine dimers, respectively, are induced in S-phase than in G_1-phase (Steward and Humphrey, 1966; Watanabe and Horikawa, 1974). This effect should not have significantly influenced the present results because most cells in the adult tissues were blocked in G_1-phase (Gelfant and Smith, Jr., 1972) and the limited number of cells in S-phase were densely covered with grains and thus excluded from repair considerations.

(b) *The amount of DNA per cell.* The amount of DNA per lung and kidney cell presumably remains at the diploid level with age.

(c) *The specific activity of internal thymidylate pools.* The levels of the deoxythymidine triphosphate (dTTP) pools in human peripheral lymphocytes from individuals of 28–83 yr were similar (Lambert *et al.*, 1979). In these non-mitotic cells, significant change in the dTTP pool did not occur with age. In the present study, we found little change in mean grain count per repairing cell as the adult hamster aged (Figs. 1b and 3b). This is consistent with lack of aging-induced changes in the specific activities of intracellular thymidylate pools. The dTTP pool of synchronized Chinese hamster ovary cells was at

a very low level (0–10 pmol 10^{-6} cells during most of G_1-phase, began a 5-fold increase at approx. 20 min before initiation of S-phase (Walters *et al.*, 1979), then decreased rapidly to its former low level as the cells exited from mitosis. Thus, dTTP pool sizes were increased only during S-phase and very early G_2 (Walters *et al.*, 1979). Since most cells in organs of adults are blocked in G_1-phase (Gelfant and Smith, 1972), and in this study were excluded from tabulations of repairing cells, dTTP pool sizes in the hamster cells undergoing repair should have been at a very low level. The similarity of mean grain counts observed in repairing cells of these tissues is consistent with the endogenous pools being low and/or similar in these tissues. The mean grain count ± S.D. of adult level repairing cells, with the number of repairing cells counted in parentheses, were: lung 13.1 ± 1.9 (2406); kidney, 16.9 ± 2.6 (1093).

(d) *The average size of repair regions.* Based upon photolysis of bromodeoxyuridine (BrdUrd) incorporated into DNA during u.v.-induced repair, Regan and Setlow (1974) found repair patches of 100 nucleotides in normal diploid fibroblasts. Setlow *et al.* (1972) found that although the numbers of repaired regions after 5–20 Jm^{-2} were different in three hamster and one mouse cell lines, the patch size in each case was about 90 nucleotides, similar to that in human fibroblasts. The per cent of dimers excised correlated well with the number of repaired regions. Using the same technique, Hart *et al.* (1979) have found similar patch sizes in fibroblasts from *M. musculus* and *P. leucopus.* These two species of mouse have similar body size but have a 2.2-fold difference in the number of repaired sites and a 2.4-fold difference in u.v.-induced UDS. Thus, patch size after u.v.-irradiation, measured by the most sensitive technique now available, has been found to be similar in the mammalian cell types studied. However, this parameter has not been determined under enough conditions to be confident that it was consistent throughout our experiments.

(e) *The number of repaired sites per unit length of DNA.* The magnitude of u.v.-induced UDS has been found to be proportional to the number of repaired sites per unit length of DNA in the wild type rodent and human cells tested (Hart *et al.*, 1979; Painter and Cleaver, 1969; Ahmed and Setlow, 1977; Peleg *et al.*, 1977). On the basis of the above considerations, it is likely that the different levels of UDS found in the hamster lung, kidney (shown here), and brain (Gensler, 1980) reflect differences in the number of repaired sites per unit length of DNA, i.e. repair efficiency.

It should be noted that u.v. radiation produces other DNA photoproducts in addition to pyrimidine dimers, so that the unscheduled DNA synthesis observed in the present experiments may reflect repair of several types of u.v.-induced DNA photoproducts.

Aging and repair

Several lines of evidence indicate that there is an increase in endogenous DNA damage during mammalian aging. An accumulation of single-stranded regions of brain and liver DNA in aging CBF_1 mice has been reported (Chetsanga *et al.*, 1975; Chetsanga *et al.*, 1977). Using a calf thymus DNA polymerase which requires DNA with a 3′-hydroxyl group facing a single-stranded region of at least 20 nucleotides for activity, Price *et al.* (1971) showed an increase in DNA synthesis with age in sections of ethanol-fixed brain, heart and liver tissues. This implies that single-stranded gaps longer than 20 nucleotides accumulate with age in these tissues. The frequency of chromosomal aberrations also has been reported to increase with age in human cells (Mattevi and Salzan, 1975).

One example of a spontaneous, repairable DNA lesion is depurination. This process

is estimated to proceed at a rate of $k = 3 \times 10^{-11} s^{-1}$ at 37°C and pH 7.4 (Lindahl and Nyberg, 1972). Several endonucleases from mammalian cells have been characterized as nicking DNA at apurinic sites, thus providing substrate for excision repair (Cleaver, 1978). Deutsch and Linn (1979) have also reported a purine "insertase" from human fibroblasts which inserts the missing base into apurinic sites.

The correlation of life span with repair capacity (Hart and Setlow, 1974) and the increase of DNA damage with age imply that accumulation of repairable DNA damage is important in determining maximum life span. Our observations that repair capacity in lung and kidney is maintained, although with fluctuation, for at least 2/3 of the maximum life span suggests that accumulation of DNA lesions with age may be a consequence of the rate of occurrence of lesions being greater than the ability of tissues to repair them, rather than to a progressive decline in repair capacity with age.

Developmental increase in repair capacity of lung and kidney

Irradiated lung cells had an increase in repair synthesis of 25-fold between days 4 and 6. Kidney cells showed an increase of 2.6-fold between days 4 and 9. These increases were due primarily to increases in the proportion of cells undergoing u.v.-induced unscheduled DNA synthesis, rather than to changes in the mean grain count of repairing cells. This suggests a developmental switch rather than a modulation. Crocker *et al.* (1970) reported a dramatic 12-fold increase of scheduled DNA synthesis in lungs of 3- and 4-day-old hamsters, indicating rapid cell proliferation, followed by a sharp decrease in scheduled DNA synthesis by day 5, indicative of a drastic slowing down of proliferation. The increase in lung repair capacity in our experiments occurred at a similar age as this rise in scheduled DNA synthesis and could have resulted from: (1) an increase in one or more subpopulations of cells capable of repair; (2) a change of repair coincident with regulation of cell cycle phase; or (3) a developmental, or programmed regulation. In rats, fibroblasts make up the majority of alveolar cells, increasing from 62% on day 1 to approx. 75% on day 4 to at least day 21 (Kauffman *et al.*, 1974). Pneumonocyte type II (granular epithelial) cells decreased from 23% on day 1 to 14% on day 4; whereas pneumonocyte type I (flat epithelial) cells remain about the same (5%). Thus, there is no obvious cell population shift which accounts for the 25-fold increase in repair capacity. Human peripheral blood lymphocytes stimulated with mitogens display higher levels of UDS induced by u.v. radiation (Bertazzoni *et al.*, 1976; Lewensohn *et al.*, 1979; Darzynkiewicz, 1971) or by *N*-acetoxy-2-acetylaminofluorene (Scudiero, 1976) than do unstimulated cells. However, the unscheduled DNA synthesis in lung cells did not decline as did scheduled DNA synthesis in rat lung cells, so that cell cycle phase appears not always to determine repair capacity. Karran *et al.* (1977) also found that replication and repair capability cound be dissociated in chick neural retinal cells. Thus, of the three possibilities listed, developmental regulation is the most probable source of the early rise in repair capacity of lung cells.

In contrast to the probable developmental turn-on of repair capacity found in the lung and kidney, we shall present evidence in the accompanying paper (Gensler, 1981) for a developmental turn-off of this capacity in brain cells. The resulting low level of repair may allow especially rapid accumulation of DNA lesions in the brain.

SUMMARY

The percentages of hamster lung and kidney cells exhibiting u.v.-induced unscheduled DNA synthesis (UDS) and the mean grain counts of repairing cells remain generally

Vol. 16 1981

similar from the age of 9 days to more than 520 days. This period comprises 2/3 of the maximum life span of this hamster strain. Repair synthesis is approx. 2-fold higher in lung cells than in kidney cells. Absence of a systematic decrease in UDS with age suggests that decline of excision repair capacity is not an important factor in normal aging. A significant increase in the proportion of irradiated lung and kidney cells exhibiting u.v.-induced UDS occurs between days 4 and 9. This may represent a developmental regulation of DNA repair.

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AGE-CHANGES IN BASIC PROTEIN CONTENTS OF LIVER AND MUSCLE OF THE FRESH WATER TELEOST, *CHANNA PUNCTATUS*

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Vol.
16
1981

INTRODUCTION

THE IMPORTANCE of the fish as an animal model for ageing studies has been emphasized earlier (Woodhead, 1978). In order to derive evidence for and against some basic concepts of ageing it was thought worthwhile to examine a single species of fish thoroughly. The choice was the fresh water murrel, *Ophiocephalus (Channa) punctatus,* a short-lived species of teleost, its life-span in nature being 4–5 years.

Our previous observations have yielded some interesting results. The loss of DNA from the brains of murrels of both sexes was confined to the sexual maturity phase (Satapathy and Patnaik, 1980*a*). On the other hand RNA and protein content and the activity of succinic dehydrogenase, a respiratory enzyme (Satapathy and Patnaik, 1980*b*) reached peak levels during sexual maturity and declined thereafter. While the age-changes in collagen characteristics (solubility in salt and acid media) of muscle followed a pattern comparable to that observed in mammals (Gantayat and Patnaik, 1980), those of skin showed an inconsistent trend.

The present studies concern age-changes in basic protein contents of two tissues, liver and muscle, having different mitotic potential.

MATERIALS AND METHODS

Animals

The fresh water murrels *Opiocephalus (Channa) punctatus* Bloch used in the present study were as described earlier (Gantayat and Patnaik, 1980). They were collected from the fish market of Berhampur during early February to mid-May. Healthy fish were kept in aquaria (size 60 × 45 × 45 cm) containing a sufficient volume of water (about 80 litres) and were maintained in the laboratory for a period of one week. As the fish are carnivorous, live earthworms were provided in adequate quantity on alternate days. The age-grouping of the fish was done as described earlier (Qasim and Bhatt, 1966; Gantayat and Patnaik, 1980).

Preparation of tissues for analysis

The fish were killed by a blow on the head. Whole liver and a piece of muscle (superficial dorsal from almost one inch behind the head region) were suspended in ice-cold distilled water. The tissues were cleaned of adherent materials, soaked in filter paper and weighed. One hundred milligrams of each tissue was used for the analysis of protein fractions.

Estimations of basic, residual and total protein contents

The estimation of basic protein fraction was almost the same as described by Malhotra and Bindal (1972).

Each tissue was homogenized [the liver in a Scientronic (New Delhi) homogenizer with a Teflon pestle for 3 min at medium speed; and the muscle with an all-glass mortar and pestle for 5 min] in 5 ml of ice-cold distilled water. The protein from the homogenate was precipitated by adding equal volume of 20% TCA and the precipitate settled after centrifugation (Remi Centrifuge, Bombay) at 1500 rev/min for 15 min. The precipitate was suspended in 5 ml of 5% TCA and the contents incubated at 70°C for 20 min to remove nucleic acids. After centrifugation the residue was saved and suspended in 5 ml of 0.2 N HCl. The containers were kept in a boiling water bath for 30 min, cooled and then centrifuged. Protein estimation in supernatant fraction was done following the biuret method of Hiller, McIntosh and Vanslyke as described by Oser (1965).

The estimation of protein in the 0.2 N HCl extract was carried out by two different methods. In method I, 1 ml of the extract was taken directly for protein estimation. In method II, the remaining solution of the extract was treated with equal volume of 20% TCA. The precipitate was settled by centrifugation at 1500 rev/min for 15 min and then dissolved in 5 ml of 3% NaOH. One millilitre of the sample was taken for protein estimation. The values of the basic protein content obtained by two different methods showed a close agreement.

The residual fraction after 0.2 N HCl extraction was dissolved in 3% NaOH (the muscle residue in 5 ml and the liver residue in 10 ml). One millilitre sample from each was taken for estimation of protein. This fraction was termed as residual protein content of the respective tissues.

The sum of the residual and basic protein contents of the tissue represented the total protein content.

The significance of age-related rising or declining trends in protein values of tissues was evaluated by calculation of correlation coefficient (r values) following the method as described in the homogenizer instruction manual (D.C.M. Data Products).

RESULTS

Age-changes in the basic protein contents

Basic protein expressed as the percentage of total protein did not show a significant age-related change in the liver of male fish (Fig. 1). But in females it showed a positive correlation with advancing age (Fig. 2).

In muscle of male fish (Fig. 3) there was a rising trend in the basic protein till the fish were aged 2^+ years, thereafter showing no further change. In the muscle of females (Fig. 4) there was a positive correlation between year class 0^+ and year class 3^+. Beyond 3 years of age there was a negative correlation with the advancing age.

Age-changes in the total protein (basic + residual) contents

The total protein content in the liver of male showed a peak at year class 2^+ and declined thereafter till year class 4^+ (Fig. 5). In females no definite trend of age-related change could be ascertained (Fig. 6).

The total protein content in the muscle of the male showed a negative correlation with advancing age (Fig. 7). But in the female, the values remained almost constant during ageing (Fig. 8).

DISCUSSION

The binding of the basic protein (histone) with DNA is known to increase with advancing age (Hahn, 1970; Thakur and Kanungo, 1980). Such an event may result in an inhibition of transcription sites of DNA. It is likely that such a repression would damage the cellular capacity for enzyme synthesis and thus the maintenance of homeostasis during old age (Hahn, 1970).

This attractive proposition needed support from a variety of experimental animal models. Much of the evidence derived so far is confined to mammalian species. There is therefore a need for such studies in non-mammalian vertebrates.

The basic protein content of a tissue does not signify the nature of its binding with DNA, but a change in its quantity during ageing is a crude measure of the amount bound in nucleoprotein. The total histone content of some tissues may not change with age (Enesco, 1968) but the histone fractions (arginine rich, lysine rich, etc.) show definite trends (Hahn *et al.*, 1969) in ageing rat tissues.

In murrels, age-changes in basic protein content show tissue and sex specificities. In males the liver did not show a significant age-related change in its basic protein content but the muscle revealed a rise. In females a distinct rise in basic protein content was evident in both the liver and the muscle of ageing murrel, but in the latter a decline occurred beyond year class 3^+.

Vol.
16
1981

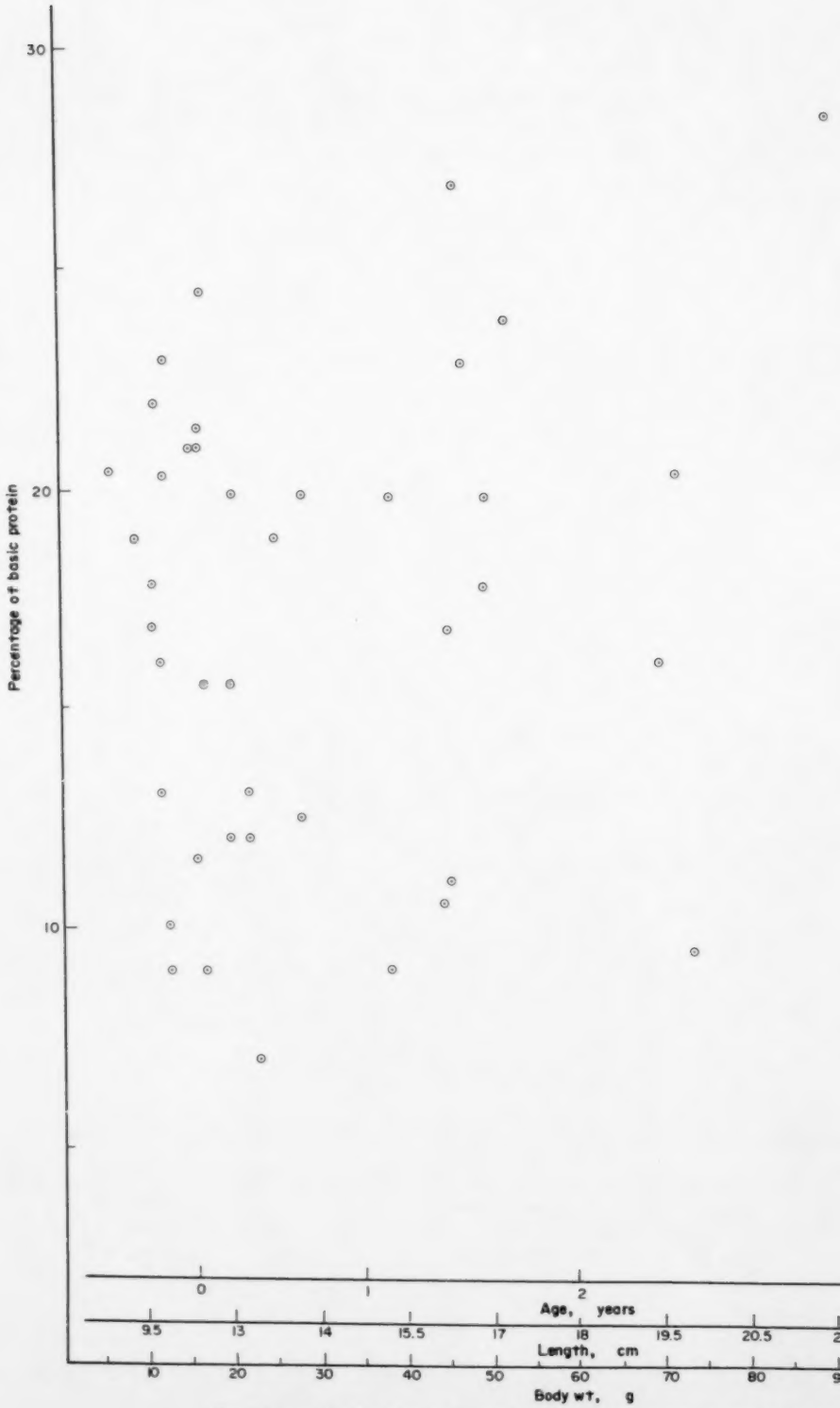

FIG. 1. Age-changes in basic protein (as percentage of total) content of the live

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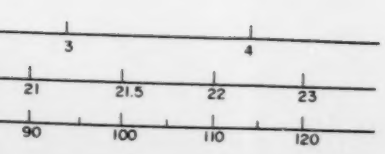


liver of male fish.

30
20
10
Percentage of basic protein
0
9.5
12.5
10
20

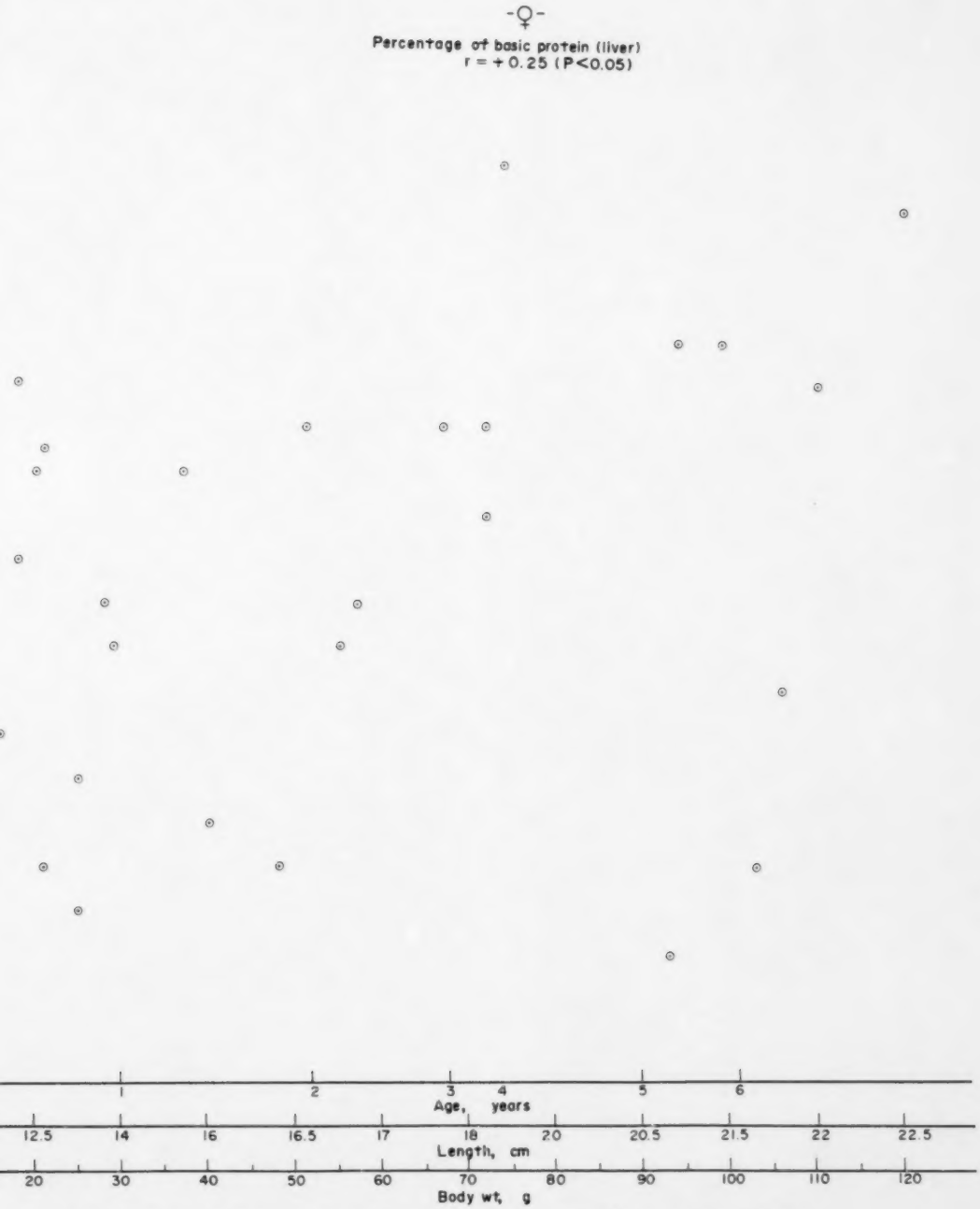


FIG. 2. Age-changes in basic protein (as percentage of total) content of the liver of female fish.

Vol
16
198

Vol. 16 1981

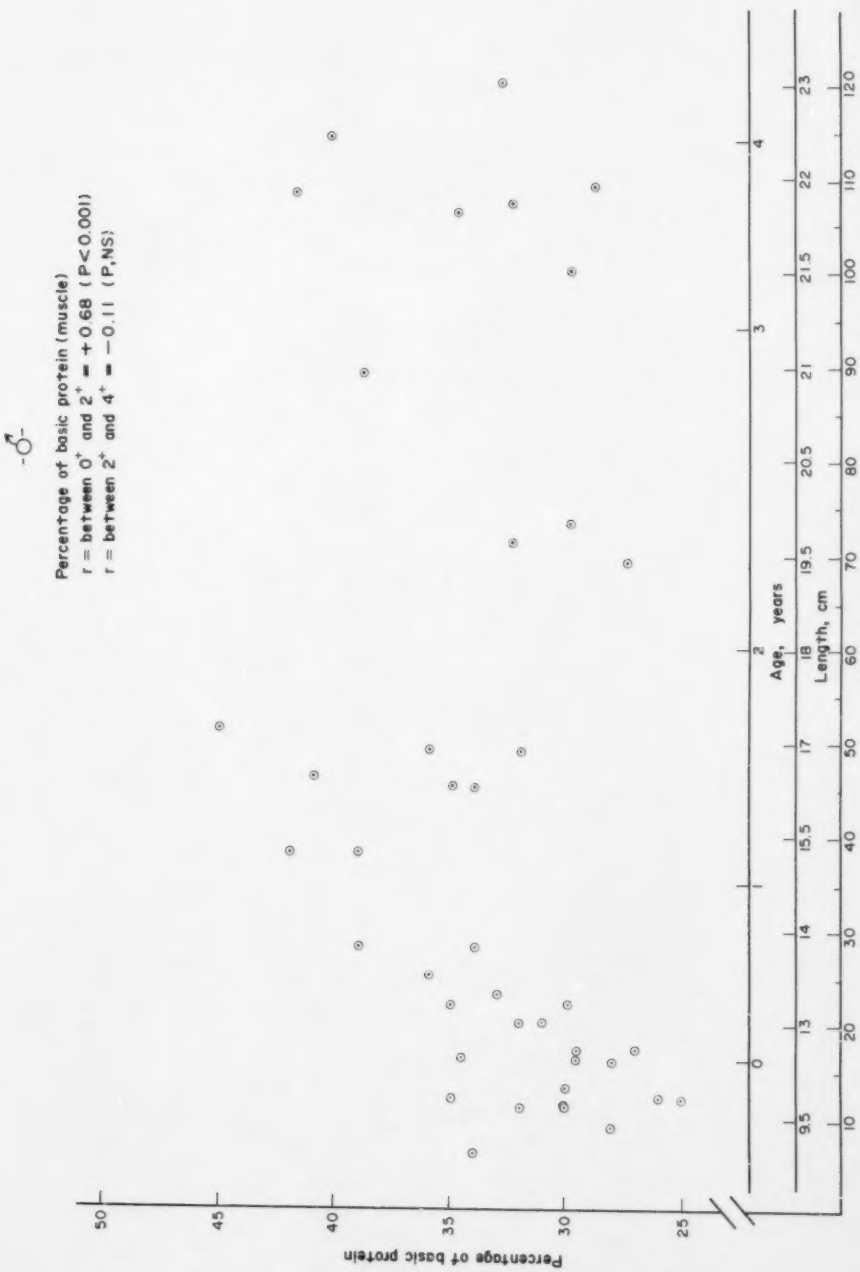


FIG. 3. Age-changes in basic protein (as percentage of total) content of the muscle of male fish.

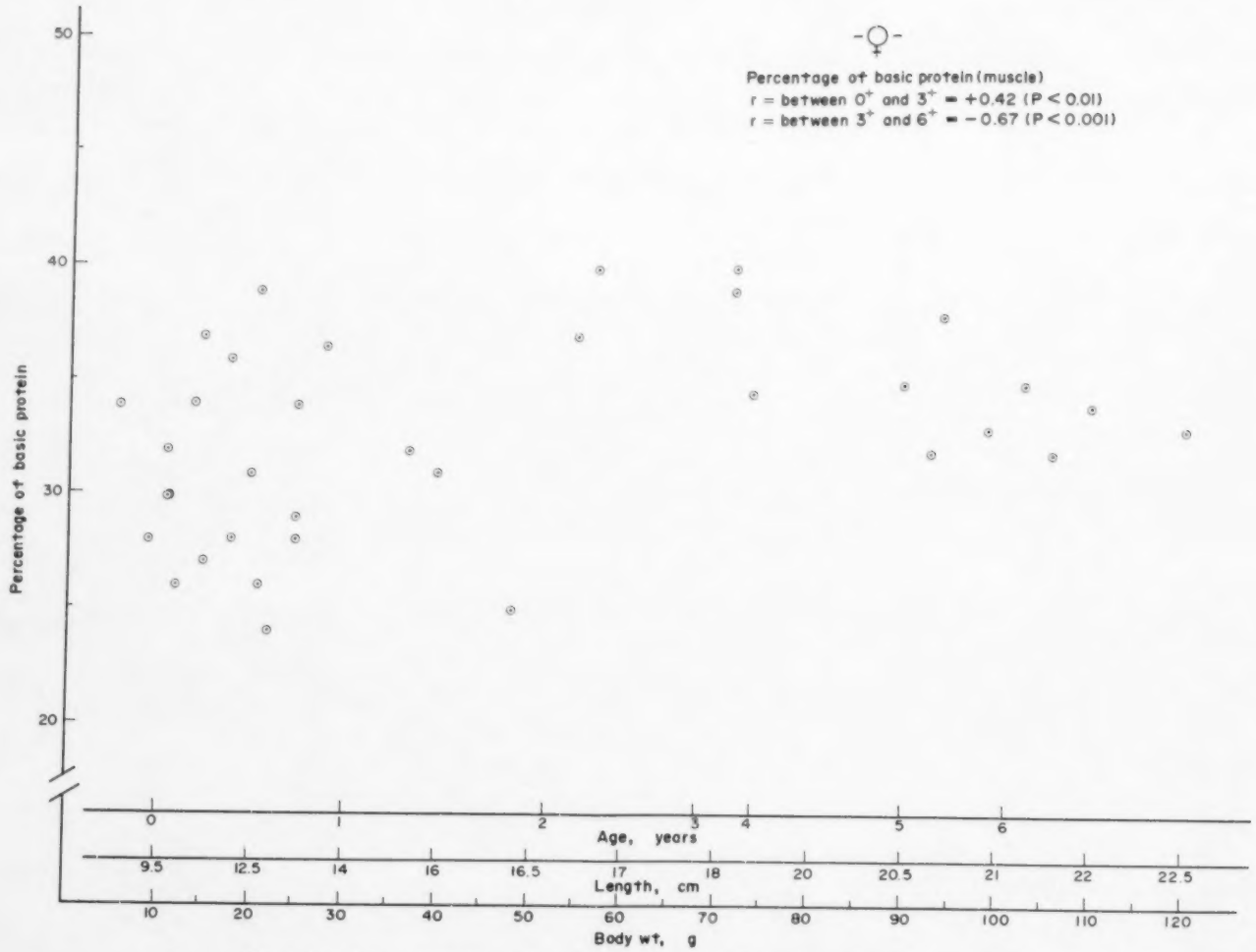


FIG. 4. Age-changes in basic protein (as percentage of total) content of muscle of female fish.

Vol.
16
1981

♂

Total protein (liver)

r between 0^+ and $2^+ = +0.24$ ($P < 0.0$

r between 2^+ and $4^+ = -0.61$ ($P < 0.0$

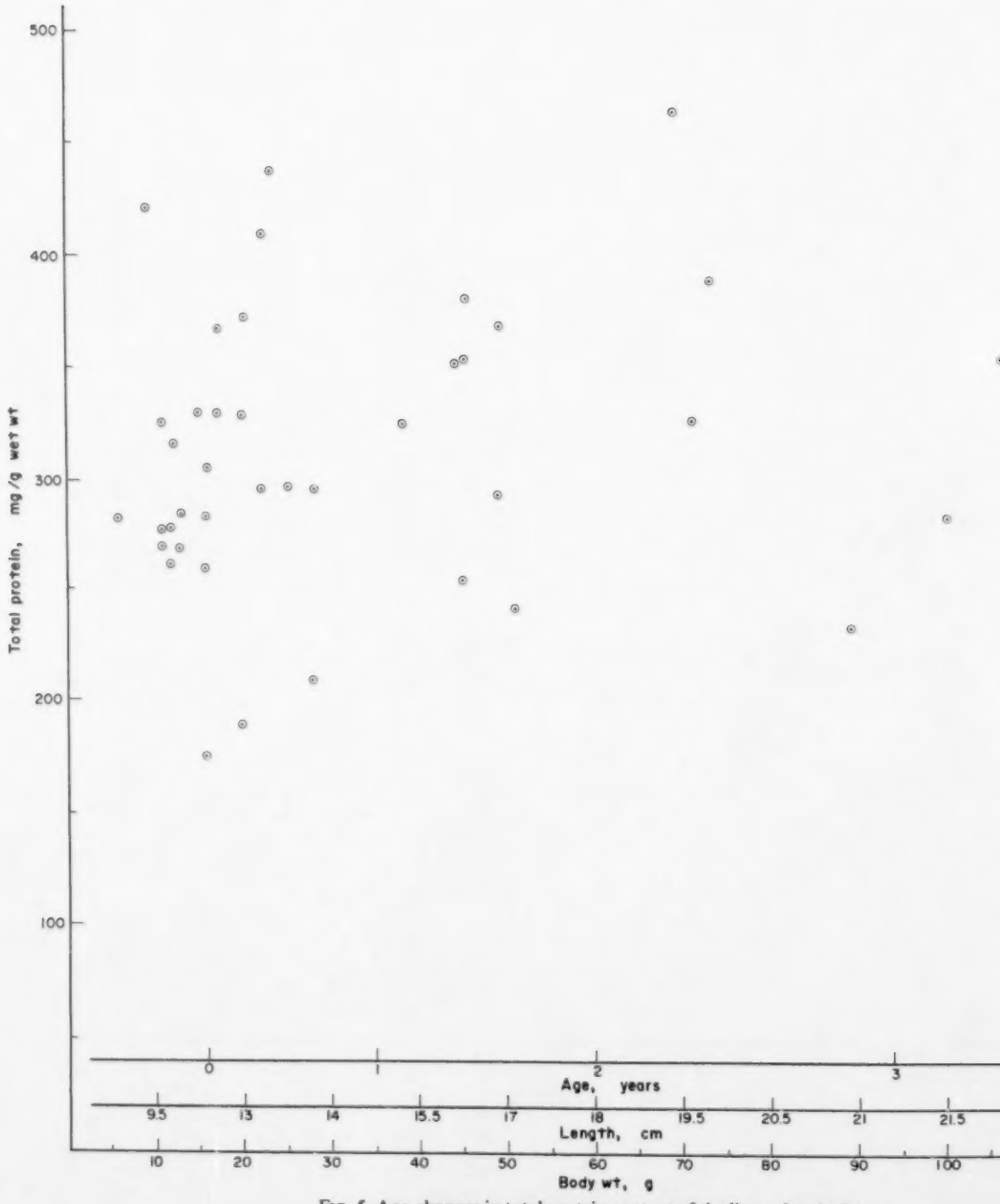
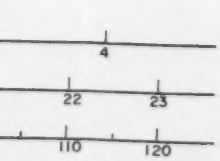


FIG. 5. Age-changes in total protein content of the liver of male fish.

P < 0.05)
P < 0.01)



-♀-
Total protein (liver)
r = between 0+ and 2+ = +0.06 (P,NS)
r = between 2+ and 6+ = +0.11 (P,NS)
500

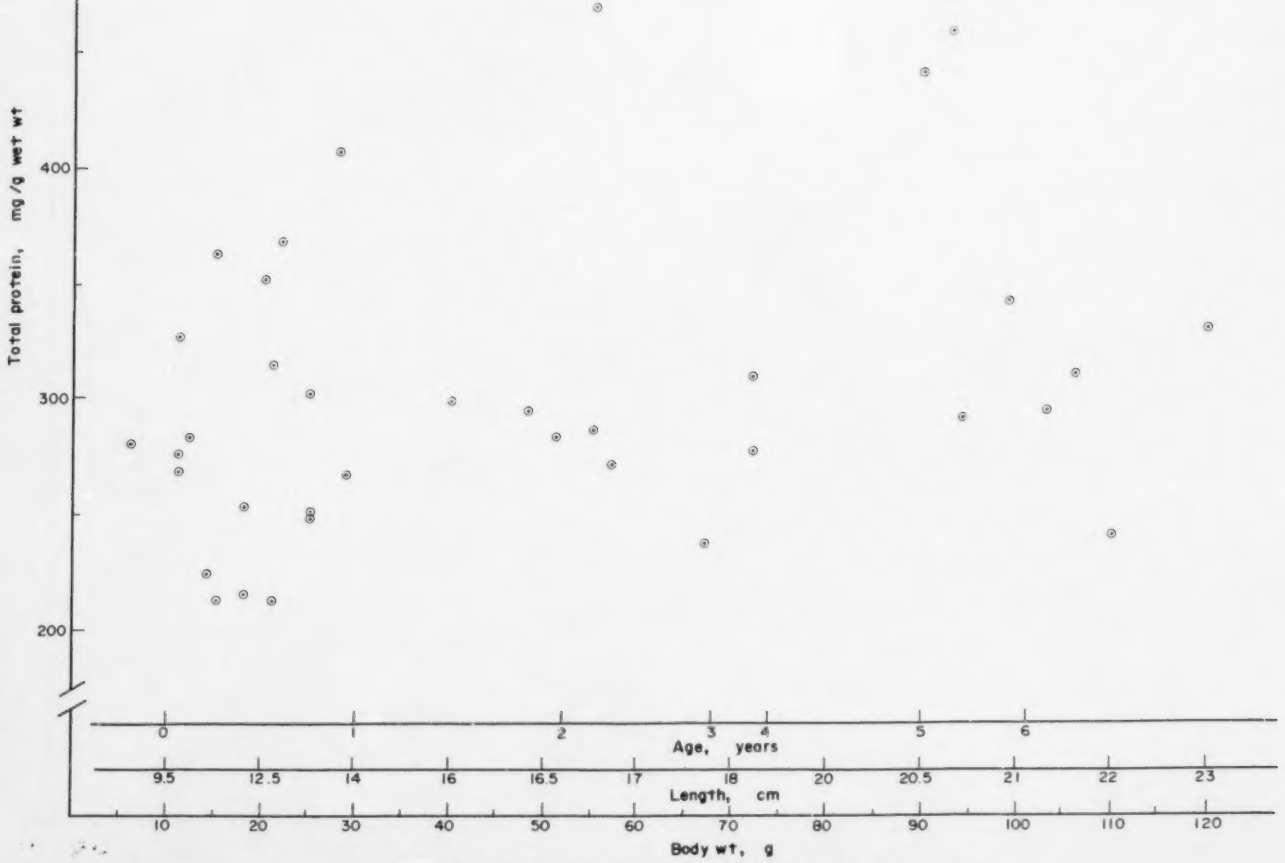


FIG. 6. Age-changes in total protein content of the liver of female fish.

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19

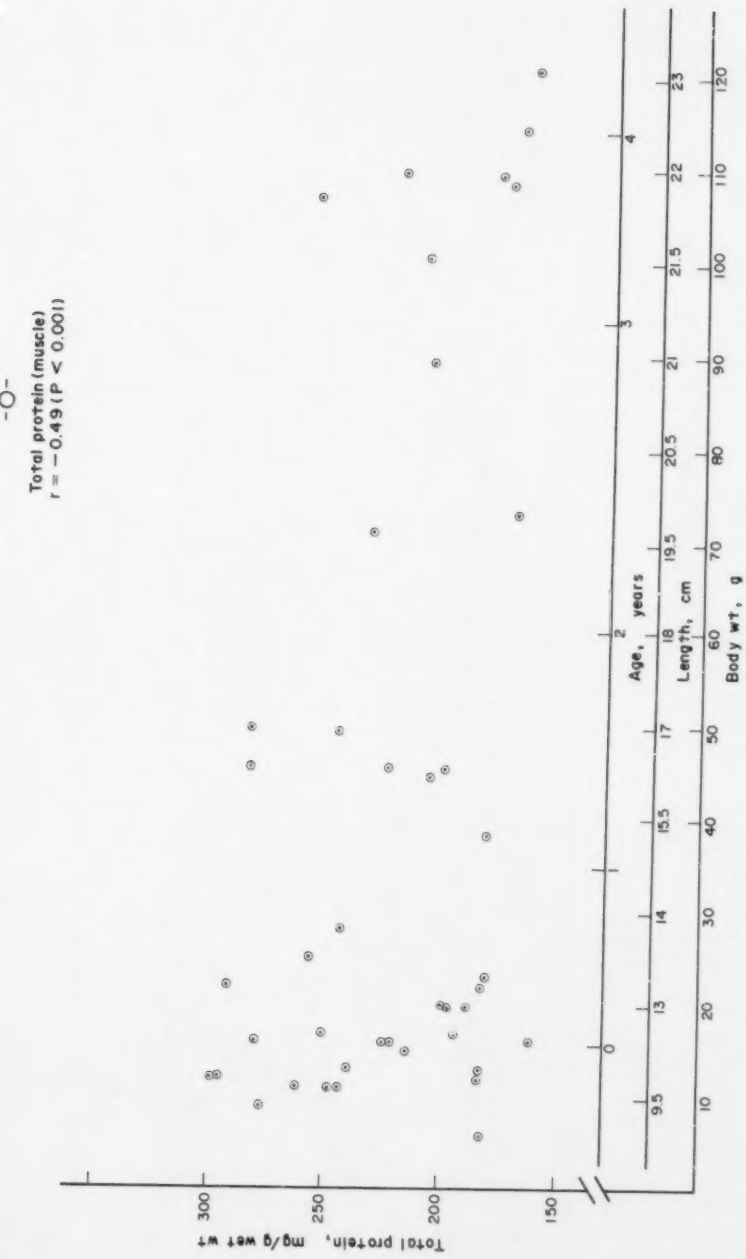

FIG. 7. Age-changes in total protein content of the muscle of male fish.

Vol. 16 1981

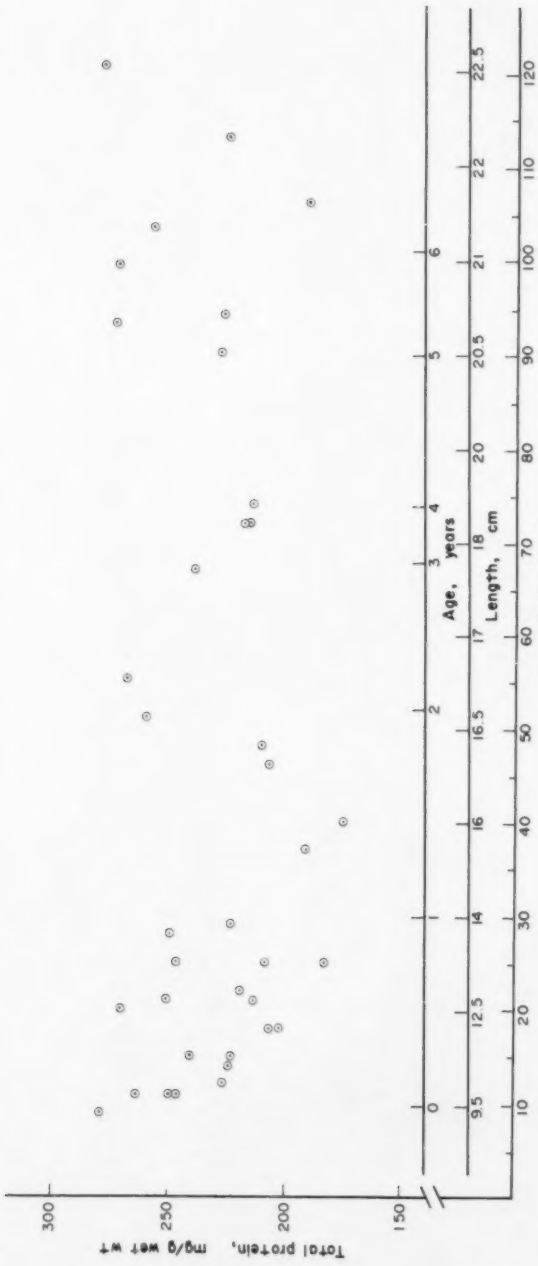

FIG. 8. Age-changes in total protein content of the muscle of female fish.

It appears that the rising trend in basic protein content in a majority of cases has no correlation with the trend of changes in total protein content of tissues of ageing murrel. While in females there was no appreciable change in the total protein content of liver and muscle, there are definite trends in males: in liver the peak level appeared in fully mature fish followed by a decline, but in muscle there was a distinct age-related trend of decline.

The sex differences in age-changes might be due to differential rates of growth. Fish of both sexes show indeterminate growth, but the rate of growth declines with age more significantly in females than in males (Qasim and Bhatt, 1966). Liver cells are reverting post-mitotically whereas muscle cells are fixed post-mitotically. Hence the observed differences between the age-changes in liver and muscle appear to be due to the differential capacity of their cells for multiplication.

On the basis of present observations we tentatively suggest that a rise in the basic protein content of ageing murrel tissues is an indicator of its increased binding with DNA in nucleoprotein. But this needs to be confirmed by *in vitro* studies using purified histone fraction. However, it appears that the role of nucleoprotein in the cellular mechanism of ageing might be of general significance in animals, as proposed by Verzar *et al.* (1972).

Vol.
16
1981

SUMMARY

The basic protein fractions and the total protein contents of liver and muscle of the murrel *(Channa punctatus)* were estimated to evaluate the effect of age. In the muscle of males and in both muscle and liver of females the basic protein contents showed a rising trend with advancing age. In males, the total protein content of liver showed a peak during maturity and declined thereafter but in muscle a continuous decline occurred with increasing age. Neither in the basic protein content of liver of males nor in the total protein contents of liver and muscle of females were appreciable age-changes observed.

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A QUANTITATIVE ULTRASTRUCTURAL STUDY OF FLIGHT MUSCLE IN *MUSCA DOMESTICA* WITH EXPERIMENTALLY LENGTHENED LIFESPANS

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Vol.
16
1981

INTRODUCTION

PREVIOUS work from this laboratory has shown a number of changes in the morphology of housefly muscle with age, the most important being the loss in the amount of mitochondrial cristae in ordered array (Turturro and Shafiq, 1979). To further validate the relationship of this mitochondrial change to aging, similar quantitative studies were performed on houseflies whose lifespan was extended by lowering of the ambient rearing temperature. Though increase of maximum lifespan by lowered temperature has been known for some time (Loeb and Northrop, 1917; Ragland and Sohal, 1975) and has been consistently achieved in metazoans by only one other means, *viz.* dietary manipulation, its mechanism of action is not known and the present study is the first report on ultrastructural morphology of a dipteran after artificial extension of lifespan.

METHODS AND PROCEDURE

Houseflies of the NAIDM strain, obtained originally from the Agricultural Research Center of the Department of Agriculture, Beltsville Maryland, where they are maintained at 26.7 ± 0.6°C (Dr N. O. Morgan, personal communication) and inbred since 1948, were used. In our laboratory, they were bred from eggs through larval and pupal stages at 25°C and were transferred at emergence to 18°C. This ambient temperature, similar to the observations of Ragland and Sohal (1975), results in the maximum lifespan being 105 days compared to the 75 days at 25°C. Flies at the age of 68 days, which would be at 80% mortality at 25°C, are only at 65% mortality at 18°C. The flight muscles of nine 68-day-old female houseflies kept at 18°C (0–18° flies) were dissected out, prepared for electron microscopy and compared to flight muscles prepared in identical fashion from nine 68-day-old flies reared at 25°C (0–25° flies) and nine 37-day-old flies (adult flies). The details of fly rearing, muscle dissection, perfusion procedure for fixation and tissue preparation for electron microscopy and the morphometric technique have been described previously (Turturro and Shafiq, 1979).

RESULTS

Figures 1a and 2a illustrate the fine structure morphology of the dorsal indirect flight muscle of the adult houseflies in longitudinal and transverse sections respectively. Figures 1b and 2b are comparable micrographs from 0–18° flies. It can be seen that the general cytoarchitecture is not much altered by rearing at the lowered temperature. The myofibrils are preserved with normal striation pattern, with similar Z-line and H-zones, the latter containing numerous glycogen granules. In the interfibrillar space there are numerous mitochondria in both the adult and 0–18° flies. The mitochondria are very close packed in the adult muscle (Fig. 2a) and tend to be less so in the 0–18° flies (Fig. 2b). Some mitochondria in the 0–18° flight muscle seem to have become less dense and have fewer cristae (Fig. 2b). In the 0–25° flies (Figs. 1c and 2c) the myofibrils are as well preserved as in the adult and 0–18° flies, but the mitochrondria have a larger

incidence of degenerative changes. In some mitochondria (Fig. 2c), the matrix is translucent and contains granules similar to those seen in the non-organelle sarcoplasm.

Quantitation of the relative volumes of the various cellular compartments from the different flight muscles is given in Table 1. The mean volume of the flight muscle fibres determined from eight 0–18° flies is 0.2043 mm^3 (cross-sectional area of 92,045 ± 13,978μ^2 and length of 2.20 ± 0.28 mm). This value is very similar to the values in adult and 0–25° flies studied previously (Turturro and Shafiq, 1979), hence the percentage volumes of the various cellular compartments of the 0–18° flies can be directly compared to those of the other two sets of flies found in the other study. The comparison shows that the 0–18° flight muscle has a mitochondrial volume very close to that of the 0–25° flies (31% rather than the 34% seen in the adult flies), a volume of non-organelle sarcoplasm intermediate to the adult and 0–25° values (although closer to the 0–25° value), a volume of sarcoplasmic reticulum equal to that of the adult and other parameters similar to the common adult and 0–25° values.

TABLE 1. MORPHOMETRY OF THE 0–18° FLY FLIGHT MUSCLE

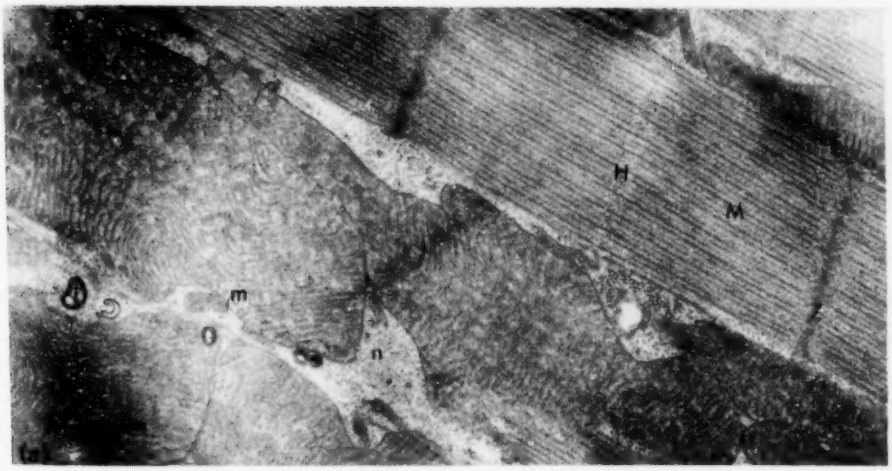
Compartment	Percentage
Mitochondrial	31.09 ± 0.46 (3116)
Myofibrillar	58.26 ± 0.49 (5838)
T-Tubular	0.98 ± 0.08 (98)
Sarcoplasmic reticulum (at dyads)	0.39 ± 0.06 (39)
Nuclear	0.54 ± 0.25 (54)
Tracheolar	2.02 ± 0.19 (202)
Non-organelle sarcoplasm	6.73 ± 0.25 (674)

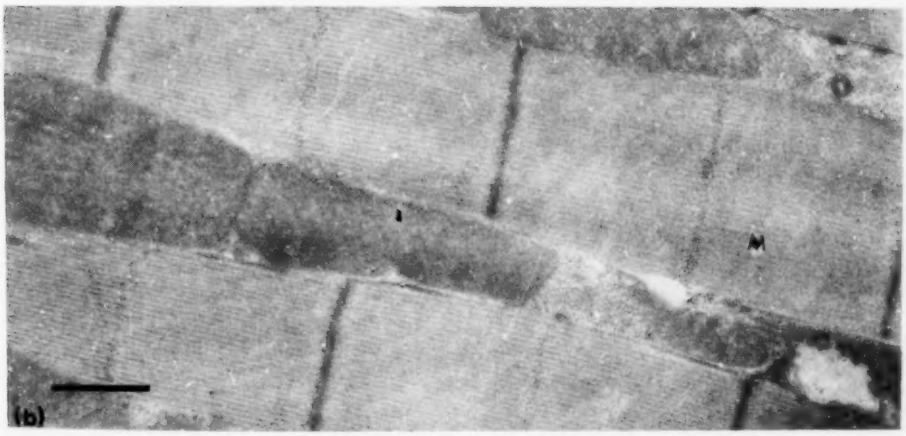
Data are presented as percentage of total cellular volume ± S.E.

Numbers in parentheses are the number of counts in each compartment taken from a grid superimposed on micrographs taken according to a randomizing procedure from random blocks from nine 0–18° female flies (six transverse and three longitudinal sections) with approximately 750μ^2 from each block.

The ultrastructure of mitochondria of adult, 0–18°, and 0–25° houseflies is seen at higher magnification in Fig. 3. The mitochondrial cristae, cut in various planes, appear in a regular array in the adult muscle (Fig. 3a) whereas they appear fewer and less neatly stacked in the 0–18° muscle (Fig. 3b). Many 0–25° muscle mitochondria have irregularly arranged cristae and have small areas where the cristae are missing altogether (Fig. 3c).

Quantifying the amount of mitochondrial cristal array volume, Table 2 gives this volume as a fraction of the mitochondrial volume and as a fraction of the whole cell volume. The decline of the cristal volume as a fraction of mitochondrial volume was 17% in the 0–18° flies (from a fractional volume of 0.345 in the adult to 0.286 in the 0–18° flies) and 51% in the 0–25° flies. Considering the cristal volume in relation to cellular volume in the two sets of old flies, the decline of cristal array volume is 24% in the 0–18° flies and 55% in the 0–25° flies. Taking the ratio of the differences in fractional volumes of cristal arrays of adult and 0–18° flies to adult and 0–25° flies, a value of 0.028/0.064, or 44% is obtained. Interestingly, this is about the same as the improvement in mortality if the effect of lowered temperature is seen as a reduction of the 80% mortality of the 0–25° flies (compared to the 50% mortality of the adult) to the 65% mortality of the 0–18° flies.





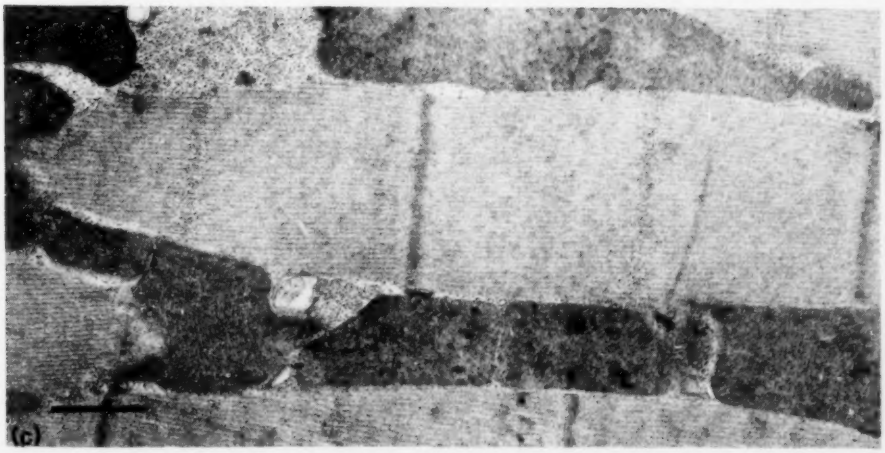


FIG. 1. Longitudinal sections of dorsal flight muscles of houseflies. (a) 37-day adult muscle. A myofibril (M) with H-zone (H) containing glycogen granules and Z-lines (Z) is seen. Mitochondria (m) have numerous cristae. n = non organelle cytoplasm; t = tracheole. (b) 0–18° flight muscle. The general architecture and myofibril structure are similar to that in Fig. 1a. (c) 0–25° flight muscle. The sarcomeres of the myofibrils are intact. Bar = 1μ.

Vol.
16
1981

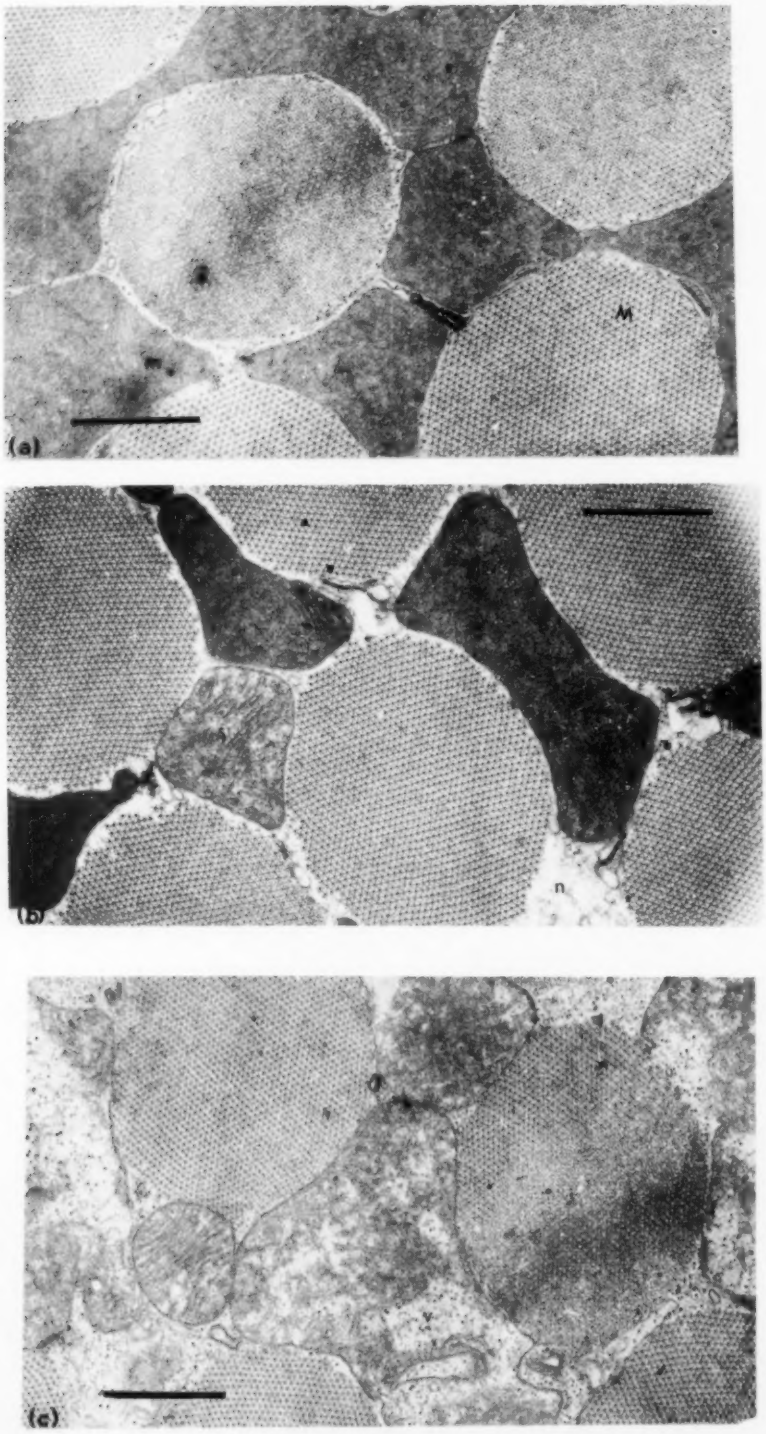
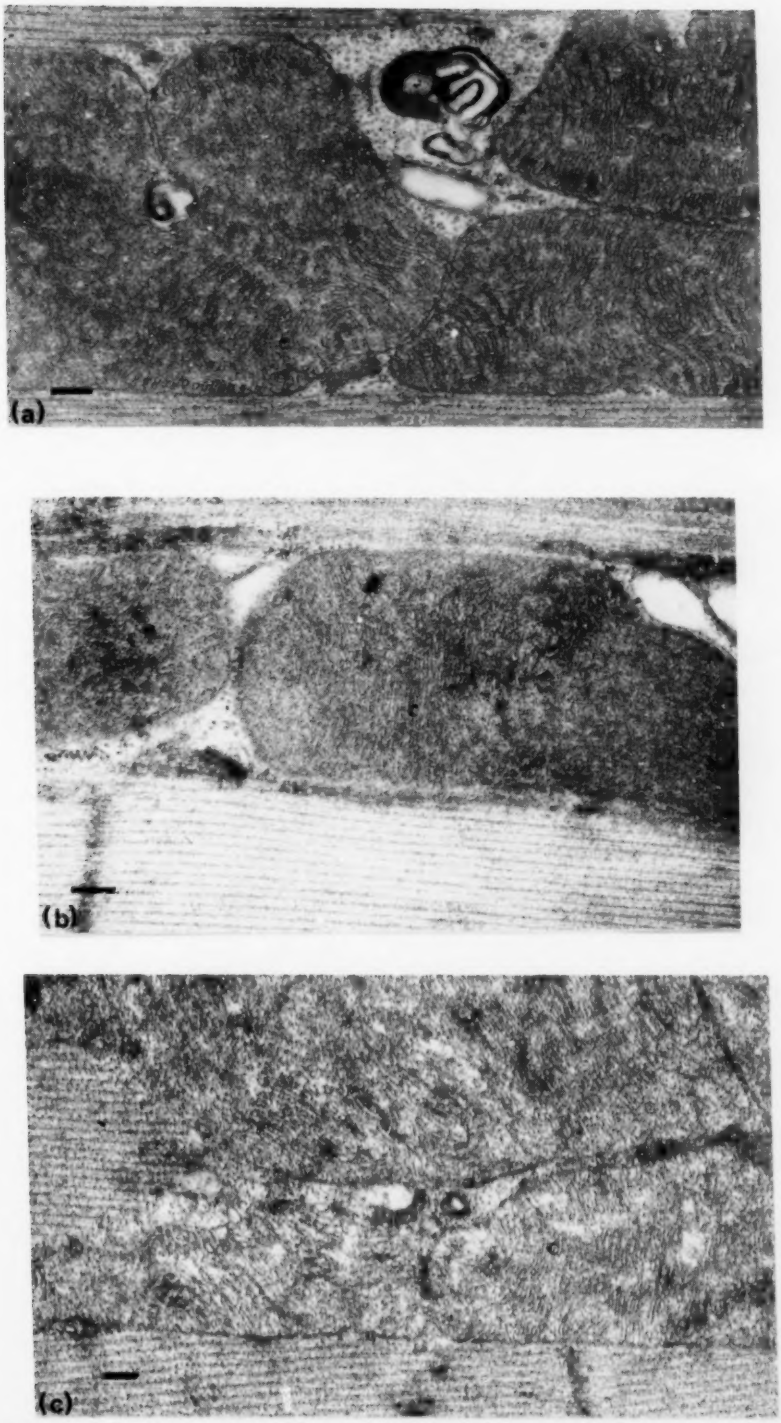


FIG. 2. Transverse sections of the flight muscles. (a) 37-day adult muscle. Myofibrils (M) cross-sectioned at levels of A, I and Z bands are seen. The mitochondria and myofibrils are closely packed. (b) 0–18° flight muscle. The packing of fiber constituents is less close than in Fig. 2a. One mitochondrion has less dense matrix than others. (c) 0–25° flight muscle. The myofibril structure is preserved but some mitochondria are translucent and contain granules. The myofibrils and mitochondria are separated by wider spaces than in either Fig. 2a or Fig. 2b. Bar = 1μ.



Vol.
16
1981

FIG. 3. Mitochondria of flight muscle. (a) 37-day adult muscle. Numerous cristae in orderly arrays are seen. (b) 0–18° flight muscle. The cristae are fairly numerous but their packing is less orderly. (c) 0–25° flight muscle. The mitochondria are translucent and the cristae are in marked disarray. Bar = 0.1μ.

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1
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TABLE 2. RELATIVE VOLUME OF CRISTAL ARRAYS

	Adult*	0–18°	0–25°*
$\frac{\text{Cristal array volume}}{\text{Mitochondrial volume}}$	$0.345\ \frac{(1173)}{(3403)}$	$0.286\ \frac{(891)}{(3116)}$	$0.169\ \frac{(506)}{(2999)}$
$\frac{\text{Cristal array volume}}{\text{Total cell volume}}$	$0.117\ \frac{(1173)}{(9989)}$	$0.089\ \frac{(891)}{(10023)}$	$0.053\ \frac{(506)}{(9625)}$

*From Turturro and Shafiq (1979).

Numbers in parentheses are counts in each region of the cell. The cristal array volume is determined from the number of points in areas in which there are at least three cristae in a stack. The total cell volume is the total number of counts in the muscle. The counting was done on the same material described in Table 1.

DISCUSSION

The 68-day-old houseflies reared at 18°C ambient temperature after emergence have a volume fraction of cristal array in the flight muscle mitochondria and in the cell, intermediate to the values of adult and 0–25° flies. This is despite similar total mitochondrial volume in the 0–18° and 0–25° flight muscle. This suggests that the mechanism which prolongs the lifespan at lowered ambient temperature concomitantly preserves the cristal stacking in the mitochondria.

An interesting point in this work is that the fiber parameters and the maturation process in the mitochondria (Sohal and Bridges, 1977) seem to be well conserved in the 0–18° flies. The major reason for placing the houseflies at 18°C only after emergence was to try to avoid interfering with the complex processes of histological differentiation and growth occurring before emergence. The similarity of the morphometric data for several parameters of the whole fibers and their subcellular organelles indicates that undue interference was avoided.

The flight muscle mitochondria, although critically important for understanding senescence in flight muscle, are probably not directly involved in the process(es) which lead to mortality in the fly and whose manipulation results in lifespan extension. However, as a model for the mechanism for functional decline of post-mitotic cells with aging, they suggest a probable source of deficits which may be operative in other fixed cells as well.

Since poikilotherms do not control their body temperature, an important consideration in the relationship of temperature and lifespan is the question of optimal ambient temperature for such species, where they are not undergoing some form of heat stress. Maynard-Smith (1963) has claimed that 25°C was the optimal temperature for reproduction in *Drosophila,* obviously an important function for the species. However, Atlan *et al.* (1976), using a variety of physiological criteria, consider the optimal temperature for their strains of *Drosophila* to be near 21°C, a result, as they suggest, of adaption to being bred at this temperature for a prolonged time. Given the history of the NA!DM strain, the flies of this strain tolerate 25°C very well and this temperature is very likely close to the optimal temperature for the strain. Therefore, it is considered here that the lifespan at 25°C is not shortened by some damaging effect of elevated temperature and that the lifespan achieved at 18°C ambient temperature is, in all probability, an experimentally extended one.

Vol. 16 1981

Most recent workers who have studied the relationship of temperature to lifespan have approached the problem by raising ambient temperatures compared to the probable optimal temperature of the strain (Hollingsworth, 1969; Lints, 1971; Nayar, 1972; Parsons, 1977). Since a variety of pathological changes can be induced by modalities, including elevated temperatures, which shorten lifespan, those which lengthen it are of greater interest to aging studies.

Several theories have been proposed relating temperature to lifespan. The rate-of-living theory (Shaw and Becaw, 1962) and the threshold theory (Maynard-Smith, 1963) and their derivatives are too vague to suggest actual mechanisms of mortality. Atlan *et al.* (1976), from a complex analysis of mortality curves, suggest that protein denaturation is important at some range of elevated temperatures. The work of Sheldahl and Tappel (1974) and Miguel *et al.* (1976) showing increased accumulation of lipofuscin in aging *Drosophila* at elevated temperatures, together with the evidence that lipofuscin deposition is a sign of damage to membranes (Tappel, 1975), suggest that early death at elevated temperatures involves membrane damage.

Although there is no direct evidence that prevention of membrane damage is the major factor in extension of the lifespan by lowering ambient temperature, the concept is not inconsistent with the data presented here. The mitochondrial inner membrane, especially its cristae, may be an important part of the control of this extension, but whether the damage is to the lipid or the protein component of the membrane is not evident. Membrane peroxidation has been considered an important factor (Harman, 1972) however, since lipofuscin does not accumulate in flight muscle, it may not be significant in this situation. Shut-off of protein synthesis (Maynard-Smith *et al.*, 1970) or some other mechanism (e.g. accumulation of damage to the mitochondrial DNA as a result of total lack of mitochondrial DNA repair (Clayton *et al.*, 1974) may be more important. Further investigations on the manner of protection of mitochondria by lowered ambient temperatures may reveal significant factors in the modulation of mitochondrial structure and functional capacity in post-mitotic cells of the aged organism.

SUMMARY

Houseflies bred from egg through larval and pupal stages at 25°C were reared at 18°C after emergence to experimentally extend their maximum lifespan. The fine structure of flight muscles from these houseflies at 68 days (0–18° flies) was compared to that of houseflies of the same age but reared at 25°C throughout (0–25° flies) and to that of 37-day-old adult flies. Myofibrillar structure was preserved in all three sets of flies. Morphometric study revealed that the total fiber volume and fractional values of a number of parameters were similar in the three sets of flies. The fractional mitochondrial volume in 0–18° and 0–25° flies were similar (31%), less than the value for the adult flies (34%). The striking finding was that the volume of the mitochondrial cristal array decreased only by 24% in the 0–18° flies, compared to the 55% decrease seen in the 0–25° flies. The greater preservation of cristal array in the muscle of 0–18° flies strengthens the idea that the cristae are important to senescence in the organism and suggests a mechanism by which lowered ambient temperature may prolong lifespan in this poikilotherm.

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Vol.
16
1981

Vol
1
19

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COMPARATIVE STUDIES OF THE AGE-RELATED CHANGES IN PROTEIN SYNTHESIS IN THE RAT PANCREAS AND PAROTID GLAND

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Vol.
16
1981

INTRODUCTION

PREVIOUS studies have reported that, as humans age, changes occur in the secretory function of the exocrine digestive glands, such as the pancreas and salivary glands. In the pancreas, a reduction in secretion has been reported in aged individuals (Necheles *et al.*, 1942). Apparently, there is no detectable change in the pancreatic stimulation by secretin (Rosenberg *et al.*, 1966). Upon repeated stimulations with pancreozymin and secretin, however, the secretion of amylase decreases in older persons, although the secretion of bicarbonate does not change (Bartos and Groh, 1969).

In the case of the salivary gland, the rate of salivary flow, basal as well as the stimulated secretion of saliva, is reduced in elderly persons (Meyer and Necheles, 1940; Bertram, 1967). Also, the enzyme content of the saliva decreases with increasing age; a reduction in the content of amylase in mixed saliva of elderly persons has been reported (Meyer *et al.*, 1937; Meyer and Necheles, 1940; Chilla *et al.*, 1974).

These reductions in the content and secretion of digestive enzymes suggest that the cellular level of the enzymes is reduced with aging, possibly due to the decline in the synthesis of secretory proteins. A reduction in protein synthesis with age has been demonstrated in many animal tissues such as the prostate gland (Mainwaring, 1968), liver (Mainwaring, 1969; Hrachovec, 1969; Kurtz, 1975; Layman *et al.*, 1976), brain (Murthy, 1966; Johnson, 1968; Johnson and Belytschko, 1969), muscle (Breuer *et al.*, 1964; Srivastava, 1969), testis (Liu *et al.*, 1978) and lens (Klethi, 1976).

We have shown previously that the cellular level of protein synthesis declines with age in the rat parotid salivary gland (Kim *et al.*, 1979; 1980; Kim, 1981). In this study we have examined the changes in the cellular level of a digestive enzyme, α-amylase, and the synthesis of protein in the rat pancreas. The results from this study have been compared with those changes detected in the parotid gland.

MATERIALS AND METHODS

Animals

Male, Sprague–Dawley rats of 2, 12, 18, 24 and 30 months of age were used and obtained from Charles River Breeding Laboratories in Wilmington, Massachusetts. All the rats were of the same strain of Caesarean derived rats, except that the older ones (12 months and older) came from the aging colony maintained by the N.I.A. at the above breeding laboratory. All rats were housed in an isolator upon arrival and allowed to acclimate for several days before beginning experiments. Food was removed from these rats about 17–18 h before the experiment, but they were allowed access to drinking water. All the rats were killed between 8 and 9 a.m. on the day of the experiments by cervical dislocation. The pancreas and parotid glands were removed from the rats and placed in a dish of ice-cold phosphate-buffered saline (PBS) for assays of α-amylase activity, and in Minimum Essential Medium (MEM) for ^{3}H-leucine incorporation studies. After removing the connective

tissue, nerves and large ducts in the central region, the glands were sliced with the Stadie–Riggs tissue slicer for incubation with the radioactive amino acid.

^{3}H-leucine incorporation studies

Tissue slices were collected in a Petri dish containing ice-cold MEM which had been gassed with 95% O_2 and 5% CO_2 and divided into groups for incubation. Each group was placed in a 25 ml Erlenmeyer flask containing 5 ml of MEM without leucine, to which 3μCi/ml of L-[4,5,^{3}H(N)]-leucine (sp. act. 47–52 Ci/mmol; New England Nuclear, Boston, Mass.) was added and supplemented with ^{3}H-leucine to a final concentration of 0.1 mM. Flasks containing tissue slices and medium were continuously gassed and kept on ice for 10 min for equilibration. After this, flasks were transferred to a water bath maintained at 37°C and agitated at about 90 c/min while maintaining the 95% O_2–5% CO_2 atmosphere. At the end of the incubation, flasks were put on ice and tissues were removed from the medium by filtering through miracloth. Tissues were further rinsed with cold PBS and homogenized. A known amount of the homogenate was removed and used to determine the total radioactivity. The remaining homogenate was precipitated with cold perchloric acid (PCA) : (final concentration, 0.32M) and fractionated to obtain PCA-insoluble protein and extract DNA, as described below. The supernatant was used to determine the ^{3}H-leucine available in the PCA-soluble pool. The PCA-insoluble pellet was dissolved in NCS tissue solubilizer (Amersham, Chicago, Il.) following the extraction of DNA. Radioactivity in acid soluble fractions and in dissolved pellets was counted in a Packard Scintillation Spectrometer. Counting rates were corrected for quenching and background with an external standard. The total leucine incorporated into proteins was calculated from the total radioactivity and the specific activity of ^{3}H-leucine in the medium.

A parallel aliquot of the homogenate was precipitated with PCA (final concentration of 0.32M) and fractionated as described by Hinrichs *et al.* (1964). DNA was extracted with hot (70°C) 0.54M PCA from the resulting pellet and the amount of it was determined according to the diphenylamine method of Burton (1956), using calf thymus DNA (Sigma, St. Louis, Mo.) as the standard.

Assays of α-amylase activity

The glandular α-amylase was extracted by an extensive homogenization of the tissue in PBS (pH 6.9; 3.0 ml/0.1 g wet weight of the gland). A small amount of the homogenate was removed, centrifuged at about 25,000 ***g*** for 10 min, and the supernatant was used to assay for its α-amylase activity following appropriate dilutions. The amylase activity was assayed by using the Amylochrome method (Roche Diagnostics, Nutley, N.J.), which is a colorimetric procedure of measuring the amount of dye released from the Cibachrone Blue F_3 GA-amylose complex upon breaking of the 1–4 bonds of the amylose by the action of α-amylase.

Light microscopy

The glands were fixed by perfusing the animal through the heart with a mixture of 1% paraformaldehyde and 1.5% glutaraldehyde buffered with 0.1M sodium cacodylate to pH 7.4. The glands were removed, sliced into smaller pieces and further fixed in the same fixative for an additional hour. The tissues were post fixed in 1% solution of OsO_4 and prepared for microscopy as described previously (Kim *et al.*, 1980). For light microscopy, 1μm thick sections were cut and stained with 1% toluidine blue for viewing.

RESULTS

The rate of incorporation of ^{3}H-leucine into proteins, as well as the cellular level of α-amylase activity among the age groups, were compared based on the DNA content of the tissue. The wet weight of the tissue was not used as the index to avoid possible fluctuations in the values due to the increase in the glandular contents of fat and other nonsecretory tissues with aging.

The incorporation of ^{3}H-leucine into proteins

When the pancreatic or parotid gland slices were incubated in MEM supplemented with ^{3}H-leucine, label was incorporated into acid-insoluble protein at a constant rate for at least 3 h (unpublished observation). Therefore, the gland slices were routinely incubated for 30 min in this study when comparing the rate of label incorporation among the age groups.

The amount of leucine incorporated into the pancreatic protein, calculated from the total radioactivity in PCA-insoluble fractions, varies with age (Table 1). The amino acid

TABLE 1. THE INCORPORATION OF LEUCINE INTO ACID-INSOLUBLE PROTEINS BY THE RAT PANCREAS

Age (months)	nmole/mg DNA/h ± S.E.
2	88.97 ± 8.13[a, b, c]
12	121.67 ± 9.20[b, c, d]
18	95.83 ± 5.78[c, d]
24	56.03 ± 4.04[d]
30	44.23 ± 2.33

[a]Value differs significantly from the 12-month value ($p \leq 0.05$).
[b]Value differs significantly from the 18-month value ($p \leq 0.05$).
[c]Value differs significantly from the 24-month value ($p \leq 0.05$).
[d]Value differs significantly from the 30-month value ($p \leq 0.05$).

Vol. 16 1981

incorporation reaches the highest level at 12 months and decreases progressively with age. The level of incorporation is greatly reduced in 24- and 30-month-old rats and amounts to only 46 and 36% respectively, of the level observed in 12-month-old rats. Although it seems that the leucine incorporation at 18 months is higher than at 2 months, the difference between the two age groups is not significant. However, the level of incorporation of leucine at these two ages is significantly higher than that detected at 24 and 30 months.

On the other hand, the amount of leucine incorporated into the acid-soluble fractions of the pancreas does not vary significantly among the age groups. There is no significant difference in the amount of leucine incorporated into the acid-soluble fractions from the pancreas of 2-, 12- and 24-month-old rats (Table 2).

TABLE 2. THE INCORPORATION OF LEUCINE INTO THE ACID SOLUBLE FRACTION OF THE PANCREAS

Age (months)	nmole/mg DNA/h ± S.E.
2	0.303 ± 0.051
12	0.313 ± 0.040
24	0.418 ± 0.037

In the parotid gland (Fig. 1), the cellular level of leucine incorporation into protein is highest in 2-month-old rats and it declines progressively with age. The level of incorporation is reduced by 21, 29, 49 and 58% of the 2 month level in 12-, 18-, 24- and 30-month-old rats, respectively.

The cellular level of α-amylase activity

The α-amylase activity was assayed by using the Amylochrome method because this procedure proved to be more sensitive and consistent than Bernfeld's technique (Bernfeld 1955). When tested by using hog pancreas amylase (obtained from Sigma St. Louis, Mo.), 1050 dye units were comparable to 1 unit of α-amylase activity which produces 1 mg of maltose from starch in 3 min at 37°C.

The cellular level of α-amylase activity detected in the pancreas is lowest in young (2-month-old) rats, but reaches the highest level among all the age groups at 18 months.

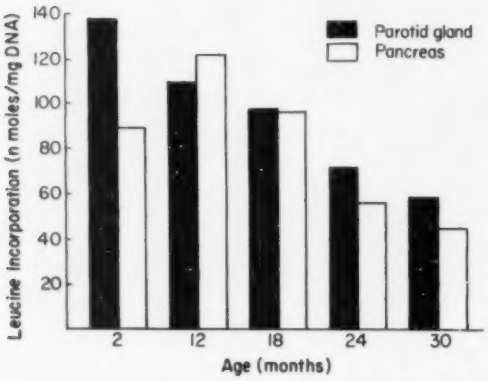

FIG. 1. Comparisons of the incorporation of ^{3}H-leucine into PCA-insoluble proteins of the rat pancreas and parotid gland at different ages. The highest level of incorporation occurs at 2 months in parotid glands and at 12 months in the pancreas. After this, the rate of incorporation declines with age in both glands.

The cellular level of the enzyme activity is significantly reduced to about 50% of the 18-month level at 30 months (Table 3).

TABLE 3. AGE-RELATED DIFFERENCES IN α-AMYLASE ACTIVITY IN THE RAT PANCREAS

Age (months)	Dye units/mg tissue ± S.E. ($\times 10^3$)	Dye units/mg DNA ± S.E. ($\times 10^6$)
2	29.6 ± 2.3[a, b, c, d]	6.3 ± 0.5[a, b, c, d]
12	67.0 ± 4.2[c, d]	15.4 ± 0.9[b, c, d]
18	77.0 ± 3.9[c, d]	20.3 ± 1.0[c, d]
24	51.1 ± 2.6	14.7 ± 0.8[d]
30	46.8 ± 4.4	11.3 ± 0.1

[a]Value differs significantly from the 12-month value ($p \leq 0.05$).
[b]Value differs significantly from the 18-month value ($p \leq 0.05$).
[c]Value differs significantly from the 24-month value ($p \leq 0.05$).
[d]Value differs significantly from the 30-month value ($p \leq 0.05$).

The microscopic appearances of the pancreas

The differences in the cellular level of α-amylase activity detected in these age groups are well reflected in the number of secretory granules present in the acinar cells. Although the number of secretory granules per cell has not been counted, it is quite obvious that the secretory acinar cells of the pancreas of 18-month-old rats (Fig. 2b) contain many more granules than those cells of the gland from 2- (Fig. 2a) and 30-month-old rats (Fig. 2c). The cells of the pancreas from 2-month-old rats contain the smallest number of granules which are concentrated around the lumen. However, it appears that the secretory lumens of the pancreas in these young rats are more dilated than those in older rats, which may indicate a high level of secretory activity in the pancreas of young rats.

One noticeable difference detectable at 24 and 30 months is the presence of fat in the pancreases of these old rats. Fat globules are mostly located at the periphery of the lobules or in the connective tissue adjacent to the lobules. However, there is no detectable difference in the amount of fat present in the pancreases in these two age groups.

Vol.
16
1981

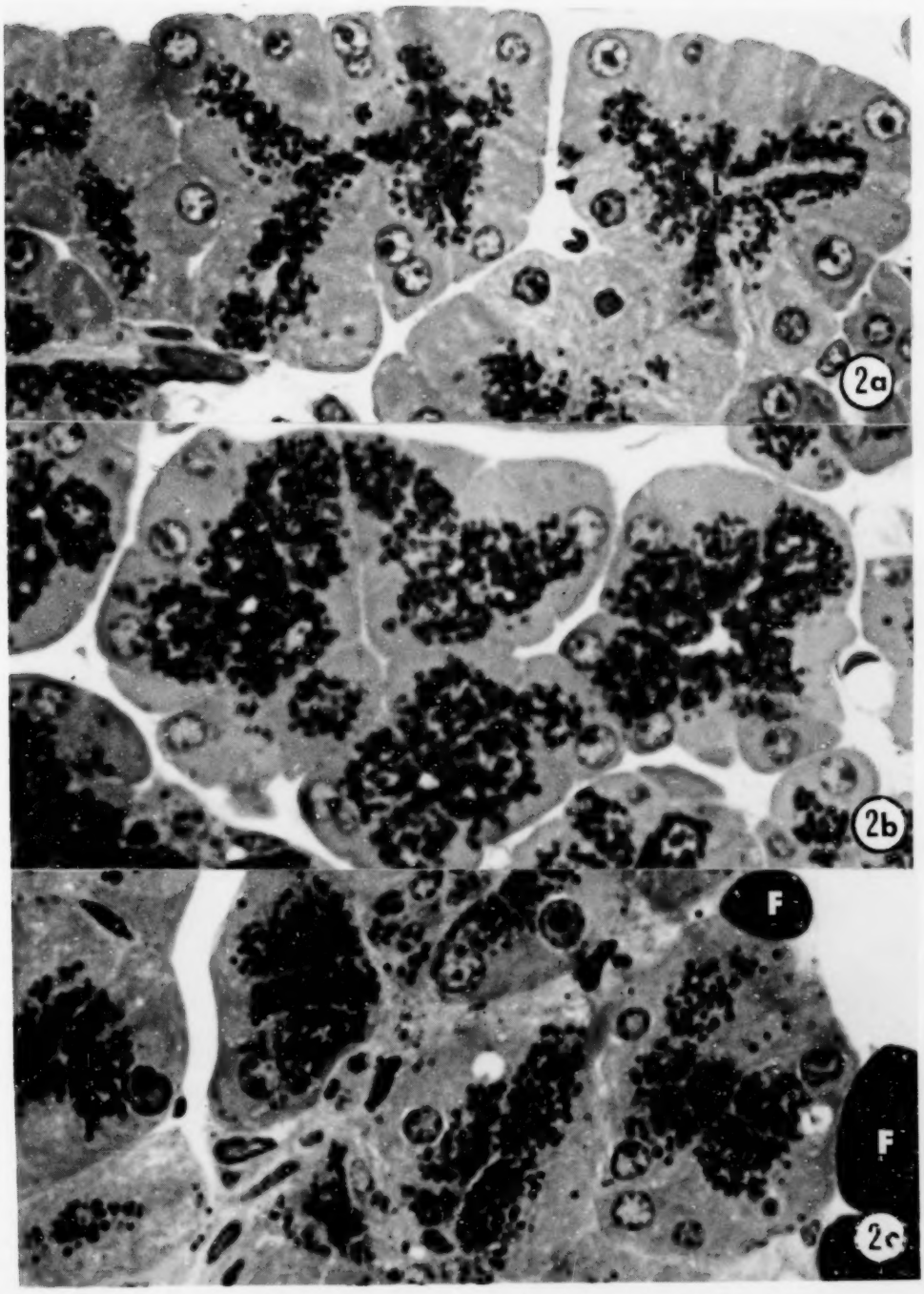

FIG. 2. Light micrographs of the pancreas from 2- (a), 18- (b) and 30-month-old (c) rats. The largest number of secretory granules is present in the acinar cells at 18 months and this correlates with the highest level of α-amylase activity detected at this age. The number of secretory granules present in the pancreas of 2-month-old rats is smallest among the three age groups. This reduced number of granules may have resulted from a high level of secretory activity as indicated by the dilated lumens (L) of the acini. Fatty tissue present in the gland at 30 months is illustrated (F). $\times 900$

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19

DISCUSSION

The gradual reductions in the rate of leucine incorporation into the acid-insoluble protein shown by the pancreatic slices of the rats of increasingly older age indicate that protein synthesis declines with age. It appears that the cellular protein synthesis in the pancreas reaches the highest level at 12 months, and thereafter it declines progressively with age. The protein synthesis declines also in parotid glands of the old rats. However, the highest level of protein synthesis occurs at 2 months (Kim *et al.*, 1980; Kim, 1980).

The decline in protein synthesis in the pancreas does not appear to be related to changes in the size of the free leucine pool, as is also the case in parotid glands (Kim *et al.*, 1980; Kim, 1981). There is no significant change in the amino acid incorporation into acid-soluble fractions of the pancreas and parotid glands from various age groups. The free amino acid pool does not appear to change with age in other organs as well. For example, the level of total amino acids (Eichholz and Buetow, 1978) and the leucine pool (Salatka *et al.*, 1971) in the livers of the rat and mouse do not change with age.

The decline in protein synthesis observed in the pancreas and parotid gland of the rat in this study is consistent with the findings of previous studies of other organs and tissues. Previous studies of *in vitro* amino acid incorporation in a cell free system have shown that the microsomes from older animals are less active in protein synthesis than those from younger animals (Breuer and Florini, 1965; Murthy, 1966; Hrachovec, 1969; Mainwaring, 1969; Srivastava, 1969; Buetow and Gandhi, 1973; Comolli, 1973; Britton and Sherman, 1975; Kurtz, 1975; Comolli *et al.*, 1976). It is uncertain as to what factors are responsible for the reduction in the rate of amino acid incorporation by the microsomes of old animals.

Vol. 16 1981

The factors responsible for the age-related reduction in amino acid incorporation have been suggested to be the inhibitory substances in the cytosol (Murthy, 1966; Hrachovec, 1969; Buetow and Gandhi, 1973; Comolli, 1973; Comolli *et al.*, 1976), or the changes in ribosomes (Mainwaring, 1969; Wallach and Gershon, 1974; Layman *et al.*, 1976). It appears that the number of active ribosomes decreases with age (Kurtz, 1978) and, thus, reduces the synthetic capacity of the cells. Recently, it has been shown that the chain elongation factor (EF-1), prepared from the rat liver and brain of 30-month-old animals, has 30–40% less activity than the same factor prepared from 2-month-old animals (Moldave *et al.*, 1979). The factor(s) responsible for the reduction in protein synthesis in the pancreas with aging remains to be studied.

The differences in the cellular level of α-amylase activity detected in the pancreas among the age groups correlate well with the differences in the number of secretory granules present in the acinar cells of these groups. Thus, it is very likely that the detected levels of α-amylase activity reflect the cellular contents of secretory proteins. Based on the amylase activity data, the highest level of secretory proteins is present in the pancreas at 18 months and the lowest level at 2 months. The secretory protein content of the pancreas at 24 and 30 months is higher than that at 2 months. Therefore, the changes in the cellular contents of secretory proteins do not parallel the changes in protein synthesis as detected by the differences in the leucine incorporation into the pancreatic protein.

However, the cellular level of secretory proteins (enzymes) probably does not depend on the level of protein synthesis alone. It is likely that the cellular level of secretory proteins reflects the net effect of the levels of protein synthesis and secretory activity of the cells. The morphological appearance of the pancreas at 2 months supports this

point. The secretory lumens of the pancreatic acini at this age are widely dilated, indicating a high level of secretory activity of the acinar cells. The dilated lumen results from the fusion of the secretory granule membranes with the apical cell membrane during the secretion of the granule contents (Amsterdam *et al.*, 1969). Then, the small number of secretory granules present in the acinar cells of 2-month-old rats are likely to be due to the high level of secretory activity of the gland at this age.

The discrepancy between the cellular level of α-amylase activity and the rate of protein synthesis also exists in the parotid gland of the same age groups (Kim, 1981). Although the highest level of protein synthesis is detected in parotid glands of 2-month-old rats, the highest level of amylase activity is detected in 12-month-old rats. Furthermore, the rate of protein synthesis is reduced by over 50%, while the content of α-amylase is reduced by only about 25% between the age of 2 and 30 months. Therefore, it appears that there is no parallel relationship between the cellular contents of secretory proteins and the rate of protein synthesis in the rat pancreas and parotid glands.

Nevertheless, it is clear that the rate of protein synthesis declines with age in both the pancreas and parotid gland, although the level of protein synthesis in parotid glands is highest at 2 months and declines progressively with age. The difference in the pattern of changes in protein synthesis observed in these two digestive glands indicates that the age-related changes in protein synthesis reflect the functional changes in each gland rather than an overall decline in the synthetic activity of the secretory cells. In fact, we have shown that the age-related decline in protein synthesis detected in rat parotid glands occurs independent of the changes in the cellular metabolic activity or the synthesis of lipids (Kim *et al.*, 1979, 1980).

SUMMARY

This study was undertaken to determine whether changes occur in protein synthesis with age in the pancreas of the rat and to compare the pattern of changes with that observed in parotid salivary glands. The rate of incorporation of ^{3}H-leucine into acid-insoluble proteins declines with age in both glands. In the pancreas, the rate of incorporation reaches the highest level at 12 months and declines by 21, 54 and 64% of this level at 18, 24 and 30 months, respectively. In parotid glands, the highest level of the amino acid incorporation occurs at 2 months and the level declines by 21, 29, 49 and 58% of the 2-month level at 12, 18, 24 and 30 months, respectively. There is no age-related difference in the rate of incorporation of ^{3}H-leucine into acid-soluble fractions of the two glands. The cellular level of α-amylase is also reduced in the pancreas and parotid gland of old (24- and 30-month-old) rats. The differences in the cellular level of α-amylase activity among the age groups correlate with the differences in the number of secretory granules present in the acinar cells of the pancreas, indicating that the level of amylase reflects the cellular content of secretory proteins. There is no detectable morphological change that parallels the over 50% reduction in protein synthesis in these glands of young and old rats.

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Vol. 16 1981

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19

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AGE-DEPENDENCE OF COLLAGEN TAIL FIBER BREAKING STRENGTH IN SPRAGUE–DAWLEY AND FISCHER 344 RATS

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Vol. 16 1981

INTRODUCTION

A CHANGE in the physical properties of rodent tail collagen fibers occurs with aging and can be measured using increased breaking time of single tail tendon fibers suspended in a concentrated solution of urea (reviewed by Harrison and Archer, 1978 and Harrison *et al.*, 1978). Some observations suggest that this measurement correlates with biological aging. For example, comparisons of *Mus* and *Peromyscus* showed that the longer lived *Peromyscus* had slower aging of tail collagen fibers (Harrison *et al.*, 1978). In addition, dietary restriction slowed the collagen aging rate when it extended the lifespan (Chvapli and Hruza, 1959; Giles and Everitt, 1967; Everitt *et al.*, 1969). Hypophysectomy also slowed the collagen aging rate (Everitt *et al.*, 1968, 1980). On the other hand, using mouse inbred strains with different lifespans, Harrison *et al.* (1978) did not find a good correlation with collagen tail fiber breaking times. The short lived strain NZB had normal collagen tail fiber aging rates, while the short lived *obese* mutant strains had faster collagen tail fiber aging rates. These studies showed that the tail fiber breaking times are not necessarily well correlated with biological aging, but the authors suggested that further tests of the relationship between collagen aging and biological aging are needed.

The present investigation is an application of the same technique to the tail fibers of two rat strains commonly used in aging research: Sprague–Dawley and Fischer 344. The results obtained, together with previously published results of Everitt *et al.* (1968) obtained using Wistar rats, imply that maximum lifespan is correlated with rate of tail fiber collagen aging in male rats.

MATERIALS AND METHODS

Animals

Both Sprague–Dawley and Fischer 344 male rats were obtained from the N. I. A. colony at Charles River. The rats were killed between 9 and 10 a.m. on a day between 1 and 3 weeks after arrival. Gross pathology and body weight were recorded for all animals. Animals with respiratory infections were not used; animals with tumors were not excluded except where noted in the data.

Assay of collagen fiber breaking strength

The fibers were tested essentially as described by Harrison and Archer (1978) submerged in buffered 7M urea at 45° ± 0.2C. The time until breaking, in seconds, was monitored using a device designed and built by Harold Thompson of Occidental College. In this device, a 2 g magnet is attached to the collagen fiber via surgical slipknot; this magnet completes an electrical circuit which drives a timing calculator. Breakage of the fiber breaks the circuit and stops the timing, leaving the total seconds until breakage on the calculator display. Unless otherwise specified, the section of tail fiber assayed was 2 cm long and the portion between 3 and 5 cm from the body. Four fibers were tested and averaged for each animal.

RESULTS

The tail fibers of male Sprague–Dawley rats between 2.5 and 30 months of age increased logarithmically in breaking strength as shown by the filled circles in Fig. 1. Each point shown is

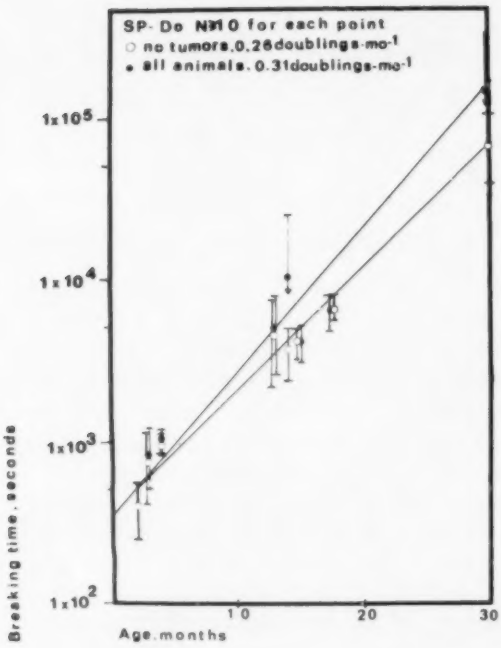

FIG. 1. Logarithmic increase in breaking time with age in collagen tail fibers from male Sprague–Dawley rats. Open circles show means and S.D.s for animals without tumors; filled circles show means and S.D.s for all animals (with or without tumors). Times were determined for 10 or more animals for each point.

based on 10 or more animals and the S.D.s are indicated. The breaking strength underwent 0.31 doublings per month for the Sprague–Dawley rats. The points shown with open circles in Fig. 1 were the averages and S.D.s for all groups of 10 or more animals of the same age without tumors. The S.D.s are sometimes greatly decreased by excluding the tumor-bearing animals (especially at 14 and 30 months), and there is a small decrease in slope, to 0.26 doublings per month. In our experience, some animals with tumors have extremely high breaking times, while others with apparently similar tumors have normal breaking times. This effect should be explored further via inoculation with defined tumor cells.

The tail fibers of male Fischer 344 rats between 3 and 24 months of age also increased logarithmically in breaking strength, a shown in Fig. 2. In this case, each point is based on 4 or more animals; S.D.s are indicated. The Fischer 344 rats exhibited a slower rate of increase in fiber strength, of 0.18 doublings per month, compared to 0.31 for Sprague–Dawley rats. The S.D.s for the Fischer 344 rats tended to be smaller than those for Sprague–Dawley rats, even though the sample sizes were smaller. This lower variance probably reflects the fact that Fischer 344 is genetically inbred, thus more homogeneous than the outbred Sprague–Dawley rats.

The fibers tested were always taken from the mid-section of the tail, between 3 and 5 cm from the body. To test for the effect of location along the length of the tail upon the breaking time, we prepared 4 lengthwise 2 cm sections of collagen fibers from 5 rats at

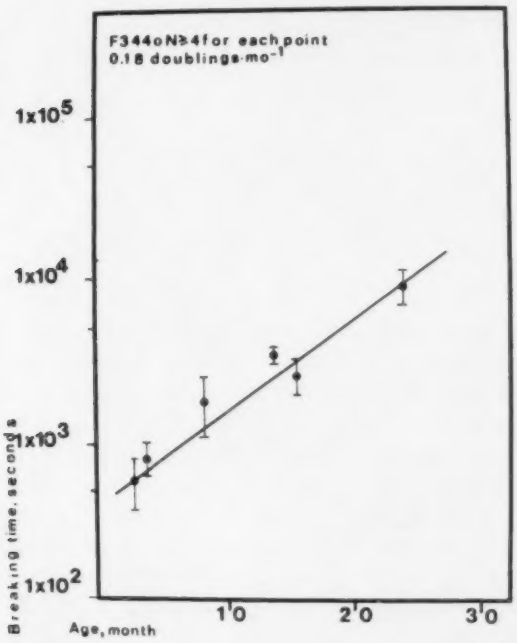

FIG. 2. Logarithmic increase in breaking time with age in collagen tail fibers from male Fischer 344 rats. Means and S.D.s of the breaking times are shown; times were determined for 4 or more animals for each point.

each of 4.5 months and 15 months old. Table 1 shows the data, which demonstrate a small tendency towards higher breaking times in the sections nearer the body. The ratio of average breaking time for the base to that for the tip was 1.2 at both ages studied.

When fibers are used routinely for controls on reported age of animals, it is sometimes necessary to store them before use. We stored fibers for up to a month in distilled water. Data showing insignificant changes in breaking time during storage are presented in Table 2. For each time interval 3 or 4 fibers from each of 5 animals were tested at 30–32 months old; 3 or 4 fibers from each of 10 animals were tested at 3.5–4.5 and at 14.5–15 months of age.

TABLE 1. COMPARISON OF BREAKING TIME ALONG THE LENGTH OF THE TAIL TENDON

Age, months	Breaking time, seconds*					Base/tip Ratio = (Section 1)/(Section 4)
	Section 1	Section 2	Section 3	Section 4	Overall	
4.5	1334 ± 238	1150 ± 125	1110 ± 92	1090 ± 186	1172 ± 184	1.2
15	5757 ± 629	6061 ± 1214	4289 ± 866	4852 ± 866	5130 ± 936	1.2

*Mean ± S.D.

Tendons were from Sprague–Dawley rats. The sections were measured starting from the body towards the tip as follows: section 1 from 0 to 2 cm, section 2 from 2.5 to 4.5 cm, section 3 from 5 to 7 cm, and section 4 from 7.5 to 9.5cm. Five animals of each age were tested.

Vol. 16 1981

TABLE 2. STORAGE OF FIBERS IN DISTILLED WATER AT 4°C BEFORE TESTING

Age (months)	Breaking time in thousands of seconds* after indicated storage period Day 0	Day 1	Day 7	Day 30	Overall average
3.5–4.5	1.14 ± 0.20	0.95 ± 0.23	1.35 ± 0.17	1.18 ± 0.20	1.15 ± 0.23
14.5–15	4.76 ± 0.70	4.25 ± 0.45	4.77 ± 0.14	5.27 ± 1.00	4.74 ± 0.68
30–32	422 ± 162	n.d.	470 ± 284	487 ± 214	458 ± 196

*Mean ± S.D.; n.d. not determined.

Tendons were from Sprague–Dawley rats, and the portion from 3 to 5 cm from the tail base was used in each case. The number of animals tested was 10 for the 3.5 to 4.5 and the 14.5 to 15 month old groups and 5 for the 30- to 32-month-old group. Three to four tendons were tested from each animal at each storage interval.

In Fig. 3 the circles show correlation of the doublings in breaking strength per month and the lifespan data reported for Sprague–Dawley (Lesser *et al.*, 1973), Fischer 344 (Coleman *et al.*, 1977), and Wistar (Deyl *et al.*, 1975) rats. The rate of change in breaking strength shown for Wistar rats was calculated from data given by Everitt *et al.* (1968). The correlation with maximum lifespan is good for these 3 strains. The triangles in Fig. 3 show a slight tendency of rate of breaking strength increase to be correlated with the 50% mortality interval for the 3 strains. The special circle and triangle symbol indicates the subgroup of Sprague–Dawley rats without tumors, which were also included in the other point for total Sprague–Dawley rats, at 0.31 doublings per month and 23.3 months until 50% mortality. The dotted arrow on this symbol indicates that this subgroup may well have a longer lifespan, although no data support that contention. The two filled triangles show intervals until 50% mortality of full fed (18.3 months) and restricted for one year, then full fed (29.4 months) Wistar rats (Deyl *et al.*, 1975). The full fed data are more comparable with the other strains, but obviously the restricted then fed animals appear to continue a correlation line between average lifespan (50% mortality interval) and rate of breaking time increase.

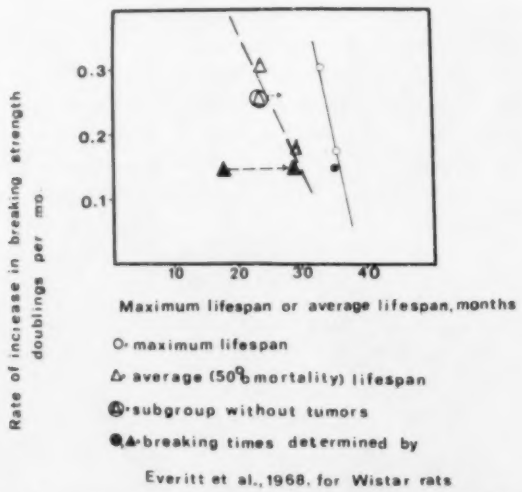

FIG. 3. Correlation of rate of collagen fiber aging with maximim lifespan and little correlation with average lifespan for three rat strains. The data for Sprague–Dawley and Fischer 344 collagen aging are from this study; the data for Wistar collagen aging are from Everitt *et al.* (1968). Lifespan data were obtained from sources given in the text. The circles show maximum lifespan and the triangles show the age of 50% mortality (average lifespan).

DISCUSSION

The data presented show that maximum lifespan in rat strains commonly used for aging research is well correlated with rate of doubling in collagen fiber breaking strength with age. This finding supports the notion that biological aging can be conveniently and reliably measured using the tail fiber breaking time technique. The main evidence against this notion is the comparison of inbred mouse strains (Harrison *et al.*, 1978). In that study, the linear portions of the breaking time vs age data were compared rather than the exponential doubling rates. It is clear from examination of their data, however, that transforming it to exponential form would not bring a strong correlation with lifespan into being, although the two strain–sex combinations with average lifespans in excess of 900 days (♀ B6CBAT6F_1, and ♀ WBB6F_1) have some of the slowest rates of doubling in breaking time. In view of the data plotted in Fig. 3 of the present investigation, it seems that maximum lifespan rather than mean lifespan of these mouse strains might correlate better with rate of increase in tail collagen breaking strength. Even this change would not eliminate the problem of the very short lived spontaneously autoimmune NZB mice, whose tail fibers behave exactly like one of the longest lived strains (CBA). The best hypothesis conserving both the correlation of collagen aging with biological aging and the inbred mouse strain data is that particular abnormalities intervene in some genotypes, preventing biological aging from running its course. This hypothesis suggests that strains whose points do not fall on or near the correlation line might die early of causes not related to normal aging. Alternative to this hypothesis is the idea that biological aging is not necessarily correlated with collagen aging.

In any case, the data show that the tail fiber breaking strength test, as carefully controlled by Harrison and Archer (1978), measures a property that increases with age in all animals studied. The present investigation shows that in rats, as in mice (Harrison and Archer, 1978), there is a slower breaking time for fiber portions nearer to the body. The ratio of breaking times of base to tip obtained for rats was 1.2, while values for mice reported tended to be higher (1.12, 1.29, 1.46 and 1.49, according to Harrison and Archer, 1978). This effect is evidently due to temperature dependence of collagen aging in mice and probably in rats as well.

If this test is used as part of a quality control program for evaluating old animals, storage of the fibers before testing may be required. The demonstrated safety of storage at 4°C in distilled water for up to a month is thus useful.

The possible effect of tumors on breaking times in Sprague–Dawley rats should be investigated further. It was clear that not all tumor bearing animals had high breaking times. For example, the data at 14 months were for 25 animals, of which 9 had tumors. Only two of the animals had breaking times above 6000 s. These two had averages of 15,547 and 129,156 s. Both had tumors. Obviously, this abnormality in breaking times was striking; for this reason, we suggest exploration of the effects of particular tumor types on breaking times.

SUMMARY

There is logarithmic increase in tail fiber breaking strength with age in males of two rat strains: Sprague–Dawley, which have a shorter lifespan, and Fischer 344. Breaking strength of Sprague–Dawley rat tail collagen fibers doubled 1.7 times as fast as those of Fischer 344 rats. The doublings in breaking strength per month were 0.31 for Sprague–

Vol. 16 1981

Dawley and 0.18 for Fischer 344 rats. These rates, plus a rate calculated from published data for Wistar rats, showed a strong correlation with maximum lifespan, but little correlation with average lifespan. The fiber section closest to the body took 1.2 times longer to break than the section nearest the tip. Storage of fibers in distilled water for 30 days was not detrimental to the determination of breaking strength.

In Sprague–Dawley rats, if rats with tumors were not considered, the rate of doubling of breaking strength per month was 0.26, as compared to 0.31 for all animals. Consideration of the data for individual animals led to the suggestion that certain tumors might well be correlated with abnormally high fiber breaking time.

Acknowledgements—We thank Richard Cutler for helpful discussions, Harold Thompson for building the device for measuring fiber breaking strength, and Susan Penn for preparing the manuscript. This work was supported by NIH Research Grant AG-00479 from the National Institute on Aging.

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Vol.
16
1981

EDITORIAL ANNOUNCEMENT

THE EDITOR-IN-CHIEF of *Experimental Gerontology* deeply regrets to announce the sudden death of our American regional Editor, George A. Sacher. The Editorial Board extends its deepest sympathy to his widow and family. Mr. Sacher's contribution to the Journal, and to experimental age studies, has been fundamental. As the leading authority on the aging effects of radiation and on the comparative biology of lifespans, he gave a paper at the Toronto meeting of AAAS shortly before his death which summarized his extensive studies, with important implications for future research. As Editor of American MSS his work for the journal was critical, unstinting, and dedicated to excellence. He was soon to have retired from Argonne National Laboratories to take up a post at the University of Chicago. A full obituary notice will appear in *Experimental Gerontology* in due course.

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TEMPORAL ORGANIZATION AND AGING IN *DROSOPHILA MELANOGASTER*

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Vol.
16
1981

INTRODUCTION

IN 1968 IT WAS proposed that aging may be due, at least in part, to the deterioration of the temporal organization of cyclic processes (Samis, 1968), although a cause and effect linkage remains difficult to support (Stewart, 1967; Samis, 1967). Perhaps of more practical importance is the possibility that aging results in changes in temporal organization which lead to functional impairment (Samis, 1978).

The importance of the temporal organization of biological processes as a determinant factor in the functional integrity of organisms (Halberg, 1969; Aschoff, 1963; Bünning, 1973) is being increasingly recognized. It appears that biological temporal organization consists of a hierarchy of clock-like cyclic processes with innate periods which approximate 24 h. These circadian "clocks" organize biological processes in time.

Evidence is also increasing which supports the notion that biological time structure is, moreover, a genetically-determined dimension of biological organization (Pittendrigh, 1954, 1958, 1961; Hastings, 1970; Konopka and Benzer, 1971, and for a review see Bünning, 1973). Biological time structure does not, therefore derive from cyclic environmental phenomena but is endogenous to the organism. In 1974 Ehret coined the term "chronotype" to connote the characteristic and genetically-determined organization of cyclic processes of an organism (Ehret, 1974) and convincing evidence exists to support this suggestion (Scheving *et al.*, 1978; Halberg and Nelson, 1978).

It should not be inferred from this, however, that time structure is not modified by environmental factors since cyclic phenomena such as those in lighting and temperature can and do cue the biological clocks. Studies on the endogenous temporal organization of different biochemical and biological processes reveal that often these processes exhibit rhythms with periods at variance from each other as well as from the 24-h day. It is now well known that this variance between processes can become organized through the levels of biological organization by virtue of what are called zeitgebers or cues, the most obvious of which are the cyclic changes in lighting within the solar day.

What may be particularly significant are recent reports which indicate that the modification of endogenous biological time structure by environmental factors can be either beneficial or harmful in terms of the organism's subsequent functioning and survival. In light of these effects, a number of researchers have recently focused on manipulation of environmental cues as a means of altering life expectancy. Hayes and Cawley (1978), based on their work with the codling moth, reported that 90° phase shifts in lighting every six days actually prolonged lifespan. Although the literature in this

*Supported by the Veterans Administration.

area is sparse, the majority of other investigators report that most unusual manipulations of environmental cues tend to decrease life expectancy.

Erk and Samis (1970) and Pittendrigh and Minis (1972) found that *Drosophila melanogaster,* when placed on a regimen of constant light and temperature, evidenced a marked decrease in life expectancy. Other lighting regimens which provide a light/dark cycle but with a period which differed from that of 24 h have also resulted in decreases in life expectancy in populations of *D. melanogaster* (Pittendrigh and Minis, 1972).

It is apparent from a review of the effects of the manipulation of biological temporal organization on survival that disorganization can lead to premature death. It is, moreover, possible that the increased rate of death which results from such manipulation of temporal organization may mimic or be due to changes which are similar to those observed in senescent deterioration.

If the imposition of abnormal zeitgebers can result in decreased longevity, presumably through alterations in biological time structure, then it becomes reasonable to ask if advancing age, with its concomitant decrease in the probability of survival, also occurs coincident with alterations in temporal organization. Furthermore, it is also reasonable to suggest that increasing age may be accompanied by changes in temporal organization which lead to a deterioration of functional capability or compensatory capacity.

Recent work by Ehret and co-workers (Ehret *et al.,* 1978), suggests that biological time structure is also subject to modification by chemical and pharmacological agents. These investigators found that periodic ingestion of certain compounds such as pentobarbital, theophylline and caffeine produce shifts in the phases of biological rhythms (Ehret *et al.,* 1978). Moreover, if ingested on a continuous basis, these compounds appear to abolish the daily or circadian rhythms previously observed.

Examined together, these findings present an interesting picture supporting our contention that changes in temporal organization may be responsible, to some extent, for those changes in declining functional capability which we label senescence. Temporal disorganization, accompanied by an increasing rate of death, at the least mimics senescent deterioration when defined in terms of survival or survival probability.

The purpose of this communication is to report the results of three studies carried out to determine if the biological time structure of *Drosophila melanogaster* changes with increasing age. Three biological functions were measured at four times of day in *Drosophila melanogaster* males from one day through to six weeks of age. The three functions investigated were: response to light, response to gravity, and catalase activity.

Drosphila exhibit a characteristic of photophilia, as well as a negative geotaxic response. It may be reasonable to suggest that changes in either of these responses could affect the adaptive capacity of the organism. To our knowledge, no studies have been done on changes which may take place in the photo response of *D. melanogaster* either as a function of time of day or of increasing age. Work by Miguel *et al.* (1976) has shown a decrease in negative geotaxic response with advancing age but, to our knowledge, the temporal distribution of negative geotaxic response has not been examined. Although the precise effects of such changes remain to be elucidated, the potential for their having a significant impact on the survival and longevity of the organisms certainly exists. Catalase was studied because of its obvious role in adaptive capacity under conditions of high peroxide loading. It is one enzyme which appears to exist in all aerobic cells which have a cytochrome system. Fortunately, it is also an enzyme which exists in sufficient excess that circadian fluctuations or age-related changes in its level need not be fatal under normal circumstances. For this reason, changes in its activity are amenable to study.

METHODS AND PROCEDURES

Drosophila melanogaster, Oregon R wild-type males, eclosed from eggs laid within a single 24-h period were used for these studies. All cultures were supplied with clean tubes and fresh instant *Drosophila* medium (Carolina Biological Supply) twice weekly. All cultures were maintained at 25°C in B.O.D. incubators equipped with 40 W fluorescent lights which were on between 0600 h and 1800 h. Complete details for culturing of the imagoes as well as their maintenance as an aging colony are described by Samis *et al.* (1971). Distinct age groups of one day, and 1, 2, 3, 4, 5 and 6 weeks were obtained by scheduled culturing of the imagoes. Production cultures were kept in 9 oz. stock bottles. Individual age-group cultures were maintained in 1.0 cm × 7.5 cm pyrex tubes.

Determinations of photophilic response were carried out using two 3.2 cm × 10.15 cm plastic *Drosophila* vials (Carolina Biological Supply), one coated with flat black paint and the other clear. The funnel of a polyethylene thistle tube was attached to the black vial so that the bulb protruded into the clear vial when the openings of two vials were brought together. To determine the photophilic response, 10 male *Drosophila* were placed in the dark vial and the clear vial quickly affixed to it, opening to opening. The assembly was laid on its side before a light source in such a way as to maximally light the clear segment of the assembly with minimal leakage of light into the darkened chamber. Five chambers at a time were easily handled and scored by one person.

The degree of photophilia exhibited was measured by counting the number of *Drosophila* which had moved into the clear chamber by +5, +10 and +15 min. Measurements were taken at 0000, 0600, 1200 and 1800 h. Ten trials were repeated for each age group at each time point.

Negative geotaxis was determined by placing 10 *Drosophila* in a 250 ml graduated cylinder, the bottom of which was covered with instant *Drosophila* medium. The *Drosphila* were knocked to the bottom of the cylinder by striking the bottom of the cylinder firmly and squarely upon a hard-rubber cushion three times. After being knocked to the bottom of the cylinder, those which ascended past the 150 ml mark within 20 s were scored. The procedure was performed on five samples of 10 male *Drosophila* imagoes of each age group at 0600, 1200, 1800 and 2400 h. The trials at 1800 and 2400 h were performed under dim red lighting (Kodak Safelight Filter 1A 10 W bulb), as this was during the dark portion of the light/dark cycle observed for these experiments.

The amount of catalase activity for single *Drosophila* was determined according to the methods of Samis *et al.* (1972), Nicolosi *et al.* (1972). Determinations were made utilizing ten samples of flies for each age group, also at 0600, 1200, 1800 and 2400 h.

Vol. 16 1981

RESULTS

Among male *D. melanogaster* from populations used for these studies age has a readily apparent and significant effect on (1) their positive phototaxic response, (2) their negative geotaxic response, and (3) their levels of catalase activity based upon total activity per imagoe.

Values for positive phototaxis, negative geotaxis and catalase activity were obtained at 0000, 0600, 1200 and 1800 h for each age group. One method of analysis employed was to combine the values obtained at all four time points and analyze them as one sample, yielding the mean indices of phototaxis, negative geotaxis and catalase activity respectively. A second method of individual time point analysis was also performed for each group of data to determine its distribution and variability over the solar day.

Table 1 presents the combined phototaxic responses for the seven age groups. Shown are the mean number of *Drosophila* entering the lighted chamber out of a total of 10; mean and standard errors of the mean are given for a total of 10 measurements. From examination of Table 1, it may be seen that the overall response to light at all three times following the initiation of exposure to light declines from one week of age to three weeks of age. Following the third week, the phototaxic response remains constant for the imagoes through six weeks of age. Closer examination of these data shows that only a slight increase occurs between the initial (+5 min) response to light and the distribution achieved at +15 min for ages one day, one week and two weeks. This appears to suggest that these organisms become maximally distributed between the dark and the illuminated chamber almost immediately. This contrasts well with the response of the older flies, suggesting a definite age-related difference in response. As can be seen, the distribution of

TABLE 1. MEAN PHOTOTAXIC RESPONSE—COMBINED DATA FROM 0000, 0600, 1200 AND 1800 H

	Age	Mean index	SEM
5 min	1 day	4.4	0.23
	1 week	4.4	0.25
	2 weeks	3.0	0.22
	3 weeks	2.0	0.17
	4 weeks	0'8	0.11
	5 weeks	0.8	0.11
	6 weeks	0.8	0.15
10 min	1 day	4.5	0.22
	1 week	5.0	0.25
	2 weeks	4.0	0.25
	3 weeks	2.8	0.21
	4 weeks	1.4	0.18
	5 weeks	1.4	0.16
	6 weeks	1.7	0.16
15 min	1 day	4.7	0.23
	1 week	5.2	0.25
	2 weeks	4.5	0.28
	3 weeks	3.5	0.24
	4 weeks	2.3	0.25
	5 weeks	2.0	0.20
	6 weeks	2.4	0.17

the 3–6 week old flies between the dark and lighted chambers changes substantially from +5 to +15 min. For the oldest group there actually is a three-fold increase across the sampling time. At the end of 15 min, however, the response of the older flies is still substantially less than the initial response of the younger flies.

From these studies data were not obtained to determine if, given additional time, the older organisms would reach distributions approximating those achieved by the younger organisms, nor can a distinction between locomotor and photophilic response be assured.

Figure 1 presents additional combined time point data for positive phototaxis, negative geotaxis and catalase activity. This graphic presentation shows even more strikingly the age-related differences in the final (+15 min) photophilic distributions achieved. Examination of these data suggest that photophilia in *D. melanogaster* reaches a maximum at one week of age, declines fairly steadily through four weeks of age, and then essentially reaches a plateau at six weeks of age. Combined time point analysis, however, does not reveal any rhythmicity or time-of-day fluctuations in photophilic behavior.

Figure 2, which presents each time point independently, shows that photophilic behavior varies significantly as a function of the time of day. Interestingly, this is particularly true for the mature but not-yet-old flies at 1–4 weeks of age. Mention should be made that the maximum degree of photophilia exhibited by the mature flies is seen at 1800 h, which is the time at which these flies entered the dark portion of their light/dark cycle. The minimum response occurs at 1200 h for one and two-week old flies. Of significance is the age-related dampening of the rhythm of phototaxic behavior which appears concurrently with the marked overall decline in the phototaxic index.

Combined time point analysis for geotaxic response is shown in Fig. 1. The degree to which each age group exhibited negative geotaxis is represented by the mean negative geotaxis index. These data show that the negative mean cumulative geotaxis response for *Drosophila* at all four times of day decreases with advancing age. A closer examination

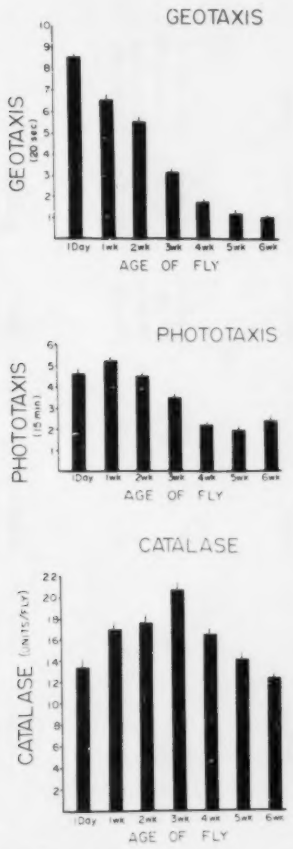

FIG 1. Mean combined time point indices of negative geotaxis, positive phototaxis, and catalase activity in Oregon R *Drosophila* males of different ages. Vertical bars represent standard errors of the means for ten individual flies samples at four different time points ($n = 40$).

shows that the maximum negative geotaxic response occurs at one day of age. The sharpest decline in the negative geotaxic response occurs by one week of age, followed by a more gradual but steady decline through four weeks of age. A low level plateau is then maintained at five and six weeks of age. These findings are reminiscent of those reported by Miguel *et al.* (1976) for which the time of day was not given.

Examination of the individual time point data for geotaxic response in Fig. 2 reveals fewer time-of-day differences in geotaxic response than in photo response, although some evidence of time-of-day differences in geotaxic response is seen at one week of age. For *Drosophila* aged two weeks through six weeks, no significant effect of time of day was found, which substantially corroborates (and complements) the findings of Miguel *et al.* (1976). Of interest is the observation that the rates at which the response diminishes are different for the different times of day sampled.

Data on catalase activity show a pattern of change occurring as the organism grows older which differs from that evidenced by phototaxis and negative geotaxis. The combined time point data in Fig. 1 show that catalase activity increases from one day of age to three weeks of age and subsequently declines from four to six weeks of age. Both rates of

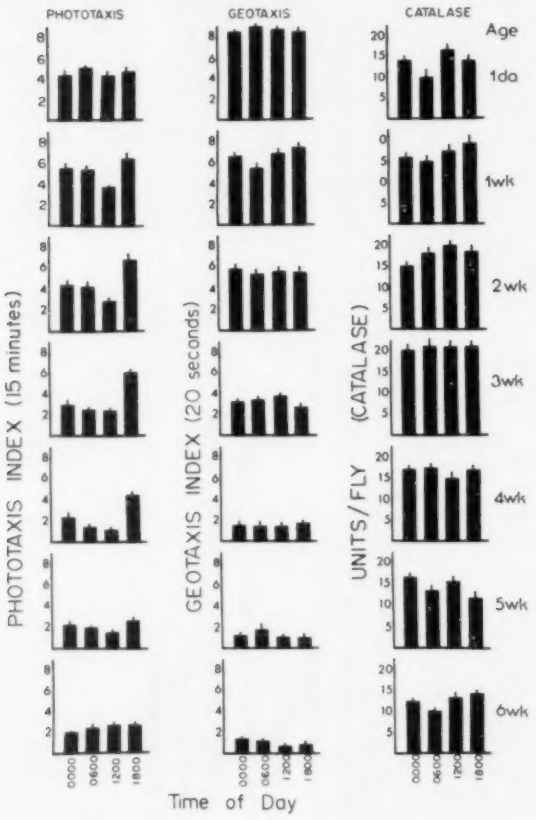

FIG. 2. Mean indices of positive phototaxis, geotaxis, and catalase activity at 0000, 0600, 1200, and 1800 h in male *Drosophila melanogaster* of different ages. Phototaxis and geotaxis indices represent the number, $n = 10$, to reach criteria within the specified time at each time point. Catalase activity based on $n = 10$ at each time point. Vertical bars represent standard errors of the means for ten individual flies samples at one time point ($n = 10$).

increase and decrease appear steady and smooth. Again, a pattern similar in most respects to results reported earlier based on data taken at a single time of the day (Nicolosi *et al.*, 1973).

Individual time point analyses of catalase activity is shown in Fig. 2. The most striking differences in catalase activity as a function of time of day occur at one day of age and five and six weeks of age. From one day of age to three weeks of age there is a dampening of rhythm accompanying an increase in activity. At the age of maximum catalase activity (three weeks) it appears that the activity of the enzyme is equal at all time points sampled. As the activity of the enzymes declines from four to six weeks of age, differences in catalase activity as a function of time of day again become apparent. At either end of the age span (one day, six weeks) the time of day of lowest catalase activity is 0600 h, the beginning of the light period. The rates at which catalase activity changes with age also differ as a function of the time of day sampled.

DISCUSSION

Although much evidence exists which shows that the deterioration of the organization of biological processes in time can have severe and deleterious consequences, temporal organization has received little attention in the context of biological aging.

The results of these studies show that awareness of the effect of time of day on the behavior of biological phenomenon is essential in the study of aging. Clearly it is important to know if cumulative or absolute activity changes with age or only its distribution in time. Moreover, it is of potentially signal consequence to determine if the function, activity or process under investigation exhibits a rhythm of discernible amplitude and period and if this rhythm is imposed by events exogenous to the organism or is simply entrained by external cyclic events. Moreover, it cannot be assumed that different processes, functions or activities will exhibit like or similar patterns of time structure.

The results reported here show clearly that in *D. melanogaster* the activities of at least two biological phenomena, catalase activity and photophilia, each show significant differences in their distribution in time as well as alterations in that distribution with advancing age.

Vol. 16 1981

A comparison of the effect of advancing age on phototaxic activity at 1200 h and 1800 h demonstrates dramatically how age effects on this behavior could be judged contradictory if measurement was to be carried out without consideration of the time of day. The most striking time-of-day differences are observed in the phototaxic activity of 1–4 week old flies whose photophilic response as observed at 1800 h was two to three times that observed at any other time point.

The activity of the enzyme catalase also exhibits a temporal distribution which changes with advancing age. An overall decline in mean activity is also observed. The time-of-day differences which were observed for catalase activity were most apparent at one day and 5–6 weeks of age.

The results of both photophilic and catalase activity measurements reported here provide clear evidence that the time of sampling must be taken into account when determining physiologic values for any age group.

The data from the studies of negative geotaxis show little variation as a function of time of day except for the group one week of age. This, however, does not necessarily mean that no time structure for this phenomenon exists. Clearly a time-of-day difference in a function or behavior with a low amplitude may require sampling over a greater number of times of day to establish its existence and its characteristics. Taking measurements at four times of day, by and large, allows for the demonstration of only those differences characterized by values showing wide excursions throughout a full day.

Both individual and combined time point analyses show that the three processes studied exhibit significant changes in their activity levels with increasing age. The decline in the absolute or overall levels of negative geotaxis and catalase activities agrees well with existing reports (Miguel *et al.*, 1976; Nicolosi *et al.*, 1973). The data show that the rates, degrees and directions of changes in both the absolute amounts and temporal distributions of these processes with advancing age are each different. If such changes are involved in a senescent process, it seems clear that the basis must be far more complex than a generalized decline of the organism's functional capabilities. Evidence to support this is particularly apparent in the data on catalase activity. Although there is an increase and then a decrease in the absolute level of enzyme activity, the low reached at six weeks of age is no lower than the level at eclosion. Clearly, the change in activity alone is not damaging to life, possibly not even deteriorative.

What may be of potentially more consequence than changes in level of amount are the age-related rearrangements of these processes in time. As these results show, activity levels of physiologic processes vary across the solar day and that temporal organization changes with advancing age. There is no *a priori* reason why changes in biological time structure should impose any diminution in functional vigour, and the occurrence of changes in temporal organization simply coincident with increasing age does not necessarily implicate such changes in either an aging process or its deteriorative correlates. However, a review of the effects of environmental manipulations on temporal organization and longevity certainly suggest that changes in biological time structure may potentially affect the functional well-being of the organism directly, or portend of other similar changes which adversely affect the organism's functional and adaptive capabilities.

Further investigation of this dimension of biological organization may reveal that simple age-related changes in absolute values or cumulative activities commonly investigated need not necessarily lead to deteriorative changes or negative consequences. Given the wide range of fluctuations which catalase may display (Nicolosi *et al.*, 1973) without compromising survival, it seem reasonable that a degenerative result of biological change may have a more complex basis than has been previously thought. It is entirely possible that alterations in the temporal distribution of physiological processes, particularly those interdependent functions juxtaposed in time, may have a far more deteriorative effect upon the functional integrity of organisms than simple changes in absolute values.

In any discussion of the means by which alterations in biological time structure may effect an aging process or generative change, the degree to which such alterations result from an endogenous genetically-determined response to events both external and internal must be considered. This becomes particularly pertinent when making interpretations of, or predictions based upon, aging and life-span data which is clearly species specific.

The work reported here demonstrates clearly the occurrence of changes in the temporal organization of phototaxis and catalase activity in *D. melanogaster* with advancing age. In addition, the importance of accounting for circadian variability when determining values for biologic functions is further demonstrated.

The findings presented here, though preliminary, demonstrate that time structure and changes in time structure should become an attended to and integral part of studies of the aging process. This is especialy so in cases where findings by different investigators appear to be contradictory or when comparative studies of behavior or function are being done.

SUMMARY

For *D. melanogaster* age had a significant effect on the levels of photophilia, negative geotaxis and catalase activity observed. Moreover, increasing age appears to affect each of these phenomena differently, each process exhibiting a distinct pattern of age-related increase and decrease in activity level. It was also found that catalase activity levels and the degree of photophilia exhibited also varied significantly as function of the time of day at which they were measured. The degree of temporal variation for each of these functions appears to be strongly age related. This suggests that the ways in which these processes are organized in time may be affected by the advancing age of the organism. It is suggested that age-related rearrangements of these processes in time affect the functional capabilities of organisms, often with severe and life-threatening consequences.

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Vol.
16
1981

Vol
16
19

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LIPOFUSCIN—DOES IT AFFECT CELLULAR PERFORMANCE?

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Vol. 16 1981

INTRODUCTION

ONE OF the most consistent morphological observations made on tissues from ageing animals (both invertebrate and vertebrate) is the presence of the intracellular ageing pigment—lipofuscin (Timiras, 1972; Schofield and Davies, 1978). This material has been noted in a variety of cells over a period of a century or more of research (see references in Bourne, 1973; Samorajski *et al.*, 1964; Strehler *et al.*, 1959). It is thought that this accumulation of pigment is irreversible and that its presence will in some way interfere with cellular function (Samorajski *et al.*, 1964; Samorajski *et al.*, 1968), but to our knowledge there is very little evidence concerning either the reasons for the accumulation of this material with age, or whether or not the presence of lipofuscin adversely affects cellular function. Various cellular organelles have been implicated in the formation of age-pigment granules with evidence for the involvement of mitochondria (Bondareff, 1957; Duncan *et al.*, 1960; Kumamoto and Bourne, 1963), various elements of the endoplasmic reticulum and the lysosomal apparatus (Brunk and Ericsson, 1972). Exhaustive histochemical, enzyme cytochemical (Strehler, 1964), and biochemical investigations (Taubold *et al.*, 1975) have been carried out on the lipofuscin granule indicating a rather complex structure with a lipid core, combined with various protein and amino acid subcomponents. There is strong evidence that lipofuscin formation can be promoted by a vitamin E deficiency in rat adrenal tissue (Weglicki *et al.*, 1968), and the role of free-radical damage in the etiology of pigment manufacture has subsequently been discussed. In addition, considerable effort is being put into the removal of lipofuscin from cells using drug treatments (Hasan *et al.*, 1974; Nandy and Bourne, 1966; Nandy, 1978; Nandy *et al.*, 1978; Samorajski and Rolsten, 1976; Schneider and Nandy, 1977; Spoerri and Glees, 1975). Unhappily, the methods reported are not quantitative and are hence very difficult to interpret in functional terms. It is also not at all clear how these drugs influence the claimed removal of lipofuscin from cells, whether it be by prevention of cellular autophagy, physical dissolution of granules, or altering the fundamental ageing patterns of the cells concerned.

This paper presents quantitative data on lipofuscin accumulation with age and its reduction during the course of an experiment on the effects of physiological stress on neuroendocrine cells from aged mice. The regions of the brain where these cells are located are the magnocellular "nuclei" of the hypothalamus; the supraoptic (SON) and paraventricular nuclei.

Cellular processes from these regions extend through the median eminence to the posterior lobe of the pituitary which acts as a reservoir for the storage of their hormone products. The cells in these regions are involved in the synthesis, transport and release of at least two peptide hormones and their associated "carrier" proteins—vasopressin, oxytocin and the neurophysins (see references in the review by Morris *et al.*, 1978). An

osmotic challenge, in the form of sodium chloride in the drinking water, stimulates the release of vasopressin from the posterior pituitary and hence, synthesis of this hormone is initiated in the SON and paraventricular nuclei (review Morris *et al.*, 1978). This technique has become standard in the study of the dynamics of hormone synthesis and release in the SON, paraventricular nucleus and posterior pituitary lobe (Morris *et al.*, 1978). In this study, the above experimental procedure was used to induce SON cell activity in animals at two ages which was then measured by quantitative morphological techniques (Weibel, 1969). The results indicate that in these cells, the presence of lipofuscin does not limit the production of hormone containing organelles (the neurosecretory granules, NSG) in either the normally hydrated or the osmotically stressed old mice.

MATERIALS AND METHODS

C57BL/Icrfa[1] mice were kept under standard conditions in a colony of ageing animals maintained by the Geigy Unit for Research in Ageing. The maintenance conditions for these animals have been described previously (Davies and Fotheringham, 1980*a*).

Electron microscopy

Virgin male mice at 6, 16 and 28 months of age were used in these observations. All operations on these animals were carried out between 10.00 and 11.00 h in order to avoid diurnal fluctuations in hormone producing activity by these cells. The animals were anaesthetised by an intraperitoneal injection of sodium pentobarbitone (60 mg/kg body weight) and perfusion took place through the heart using a small peristaltic pump with a flow rate of approximately 2 cm^3/min (Davies and Fotheringham, 1980*a*). The perfusion procedure, together with the fixation, dehydration and embedding schedules have been described previously (Davies and Fotheringham, 1980*a*).

Osmotic loading

Only two sets of animals were used in this experiment; these were aged 6 and 28 months. Groups of these animals were administered an osmotic challenge consisting of 3% sodium chloride in the drinking water for a period of up to six days, with sampling of control and experimental groups at 1, 2, 3, 4 and 6 days. On each day one control (i.e. normally hydrated) and three experimental animals were killed and prepared for electron microscopy. The data in this paper refer to the six day salt loaded animals exclusively. In addition, a group of normally hydrated animals at 16 months of age was included in the experiment. All details of the experimental procedure used are described elsewhere (Davies and Fotheringham, 1981*a*, *b*).

Stereology

All details concerning the sampling of the SON cells and the quantitative methods used to assess the electron micrographs have been published previously (Davies and Fotheringham, 1980*a*, *b*, *c*). The relative volume fractions of lipofuscin and NSG were determined in the SON cells at the ages described.

Statistics

Observations from the individual animals within the control groups at each age were pooled to produce a mean of means for each age group. An analysis of variance was used to test between the three control groups. Subsequent testing between all possible pairs of observations was carried out using a multiple comparisons test (Scheffé test) with probability values set at 0.05 and 0.01 from *F*-distribution tables (Winer, 1971). Testing between the control and experimentally stressed animals employed the Students *t*-test. In addition, non-parametric tests were also used to assess the differences between the control and experimental groups with essentially similar results. All tabulated data are presented in the parametric form for convenience.

RESULTS

There is a progressive increase in the relative volume fraction of the SON cells occupied by lipofuscin between 6 and 28 months of age (Table 1). However, the only significant increases recorded by the multiple comparison test were between 6 and 28 months ($p < 0.01$), and 16 and 28 months ($p < 0.01$) of age. These observations indicate that the main period of lipofuscin accumulation was in the latter part of the lifespan.

The relative volume fractions of the NSG in the SON cells are given in Table 1. There is a significant decrease in the relative volume of NSG recorded between 6 and 16 months

TABLE 1. THE INFLUENCE OF AGE ON THE PERCENTAGE VOLUME FRACTION OF LIPOFUSCIN AND NSG IN NEURONES OF THE SON

Age (months)	n	Lipofuscin	NSG
6	5	1.02 ± 0.10	0.295 ± 0.04
16	4	1.43 ± 0.23	0.213 ± 0.02
28	5	2.46 ± 0.31	0.316 ± 0.03

Data presented as pooled means for n animals. Mean ± 1S.D. See text for statistical comparisons.

of age ($p < 0.01$) followed by a significant increase during the period 16 to 28 months of age ($p < 0.01$). However, there is no difference in the values recorded for 6 and 28 months of age.

There were no significant differences in the relative volumes of the cytoplasm, nuclei and mitochondria over the period investigated (analysis of variance, $p > 0.05$), although a decrease in the relative volume of the golgi-rich zone was recorded between 6 and 16 months of age (multiple comparison test, $p < 0.05$), followed by an increase between 16 and 28 months ($p < 0.05$). There were no significant differences in the data for the relative volume of golgi at 6 and 28 months of age.

Marked changes take place in the ultrastructure of SON cells of both young and old mice under osmotic load and these have been described elsewhere (Davies and Fotheringham, 1980*b*). Only the changes in the relative volumes of SON cells occupied by lipofuscin and NSG are reported in this paper. After six days of osmotic stress there is an approximate 50% reduction in the volume of the SON cells occupied by lipofuscin at both ages (Table 2). However, there is no significant difference in the relative volume of NSG in these cells at either age under these conditions (Table 3).

TABLE 2. THE INFLUENCE OF BOTH AGE AND OSMOTIC LOAD ON THE PERCENTAGE VOLUME FRACTION OF SON CELLS CONTAINING LIPOFUSCIN

	Age (months)		
Treatment	6	28	p
Control ($n = 5$)	1.02 ± 0.1	2.46 ± 0.31	0.001
Salt-load ($n = 3$)	0.58 ± 0.038	1.03 ± 0.11	0.001
p	0.001	0.004	

Data presented as pooled means for n animals. Mean ± 1S.D. Probabilities derived from Students t-tests (two-tailed).

DISCUSSION

It is generally claimed that lipofuscin is a waste-product which accumulates within cells and that its presence is an indicator of cellular inefficiency. There is some suggestion that the accumulation of lipofuscin in neurones in autopsy specimens of the human central nervous system is positively correlated with a reduction in cellular RNA content (Mann *et al.*, 1978). In this case there is at least a potential decline in cellular protein

synthetic function with age and pigment accumulation. However, a recent paper (Anthony and Zerweck, 1979) has indicated that neurones in the auditory cortex of old mice contained less RNA than those of young mice, but upon auditory stimulation neuronal RNA values were not significantly different between young and old animals. A recent paper by Brizee and Ordy (1979) showed that there were large increases in the amount of lipofuscin present in neurones from selected areas of the hippocampus and visual cortex of the rat. In addition, there is a strong intercorrelation between the accumulation of age-pigment, reduced neurone density and reduced short-term passive-avoidance retention behavior with age.

TABLE 3. THE INFLUENCE OF BOTH AGE AND OSMOTIC LOAD ON THE PERCENTAGE VOLUME FRACTION OF SON CELLS CONTAINING NEUROSECRETORY GRANULES

	Age (months)		
Treatment	6	28	p
Control ($n = 5$)	0.295 ± 0.04	0.316 ± 0.03	0.59
Salt-load ($n = 3$)	0.34 ± 0.03	0.34 ± 0.07	0.95
p	0.17	0.53	

Data presented as pooled means for n animals. Means ± 1S.D. Probabilities derived from Students t-tests (two-tailed).

In many of the investigations published so far it is implied (if not always stated) that age-pigment is detrimental to cellular function. However, to our knowledge, no one has attempted to critically evaluate this proposal. Indeed, others have recently voiced the same concern (Siakotos *et al.*, 1979). In this paper we confirm a significant increase in lipofuscin with age, but the relatively small volume of the SON cells occupied by this material leaves considerable doubt as to its potential for the disruption of cellular activity. Indeed, using the criterion of the volume of NSG in the SON cells as an indication of the functional potential at different ages, we can demonstrate a decrease in the NSG volume content between young mature and middle aged mice, with no significant increase in the volume of lipofuscin over this period. There is a significant increase in the relative volume of the NSG between mature and senescent mice, but also a significant increase in the lipofuscin content of the cells. In these cells therefore, there is no apparent connection between lipofuscin content and hormone content (as determined by these quantitative ultrastructural parameters). However, an elevated lipofuscin content does not preclude finding similar volumes of hormone granules in old as in young cells. In addition, the SON cells from old animals undergoing osmotic stress contain similar relative volumes of NSG to those from young mice. The decline in the volume fraction of lipofuscin under conditions of salt-stress suggests that these structures are not being maintained in some steady-state and are being allowed to deplete from the SON cell perikaryon. Hence active synthesis and turnover in the normally hydrated animal would seem highly likely. The reason for the decline of the lipofuscin organelles in osmotically stressed mice may be that cellular activity under these conditions is switched to a programme with NSG production as the "prime" objective. The suggestion has been made (Davies and Fotheringham, 1980*c*) that this programme is protected late into the lifespan, possibly at the expense of other cellular response and maintenance schedules. However, it should be noted that

measurements made on the size and numbers of NSG per unit volume of SON perikaryon and posterior pituitary neurosecretory nerve endings and swellings indicate that rather subtle changes are taking place in these structures also (Davies, Fotheringham and Wilkinson, in preparation). A critical, qualitative and quantitative evaluation of other cellular organelles in the SON cells of osmotically stressed old animals is presented elsewhere and it is evident that there are declines in the structure and function of a variety of cytoplasmic components under these circumstances (Davies and Fotheringham, 1980*b, c*).

As to the origin of these organelles in SON neuronal perikarya, a few comments can be made. Siakotos *et al.* (1979) proposed a modified Novikoff sequence (see Siakotos *et al.*, 1979 for references) which involves the fusion of lipid globules or cytolysosomes with primary lysosomes and microperoxisomes. This structure eventually becomes the residual body of the autophagic sequence, termed the "mature" lipofuscin granule by Siakotos *et al.* (1979). Siakotos bases his schema on sedimentation velocity isolation of lipofuscin from human brains with an age range of 0.36 to 78.8 years. In the age groups up to approximately 17 years of age the lipofuscin pigment is composed of electron lucent globular areas at first, with an increasing electron dense component added with time. After this age, the electron dense component increases. In the mouse SON cells (Davies and Fotheringham, 1980*b, c*) there is a proportionally greater increase in the vacuolated lipofuscin organelle with age. The source of the lipid globules or cytolysosomes in the proposed sequence suggested by Siakotos *et al.* (1979) is not stated. In the SON cells of young mice lipid globules arise during periods of osmotic stress (Davies and Fotheringham, 1980*c* and references therein) but to a significantly lesser extent in the older animals. In addition, it is possible to see these lipid bodies in young normally hydrated animals, but it is a *very rare event*. We have not encountered these lipid organelles in old mice of the same physiological status. In addition, using serial sectioning techniques at the electron microscope level, we have never seen a close association between lipid droplets and electron dense lysosomal structures in SON cells, even though the conjunction of these two structures was an attractive possibility in the formation of the vacuolated lipofuscin granule. Since our data suggest lipofuscin turnover, we can only conclude that the presence of these electron lucent vesicles is not a major source of potential lipofuscin material in these cells. Subsequent studies of osmotically stressed animals which have been allowed to recover and rehydrate may produce valuable information on the formation of the vacuolated lipofuscin granule.

In conclusion, the SON perikarya of ageing mice accumulate lipofuscin but this does not necessarily lead to a decrease in the volume content of NSG in these cells and there is no detectable decline in what has been termed the "prime" function of the SON cell in either normally hydrated or osmotically stressed mice. There is a period during the lifespan when the relative volume of the NSG decreases in the SON perikaryon, but no reasons for this are immediately apparent. Similar rather paradoxical observations were made on intranuclear inclusions in the nuclei of SON cells from aged female mice. In this case, there was a continuous increase throughout the lifespan until, in the oldest age group (32 months) there was an unexpected decline (Fotheringham and Davies, 1980). This was discussed with respect to selection of the "fittest" animals among extreme old age groups and may be applicable in this case (Fotheringham and Davies, 1980). No conclusions may be drawn with respect to other cell types because of the widely disparate relative volume content of lipofuscin in these various cells. For example, a recent publication

Vol. 16 1981

(Brizee and Ordy, 1979) reports the content of lipofuscin in neurones of the hippocampal CA1 zone as being 35.58% and in those of the visual cortex as 30.65% in 29-month-old rats. However, the results obtained in this study cast serious doubts on the role of lipofuscin as being deleterious; it seems more likely that it is an indication of active dissolution of redundant cellular organelles and as such is some form of protective mechanism for the cell. It is also evident that the accumulation of lipofuscin as an age-specific biological parameter (ASBP, as discussed in Donato *et al.*, 1979) is related to increasing intracellular damage with age. Therefore, it is of the utmost importance to understand what is happening with respect to the various drug treatments claiming to reduce lipofuscin accumulation. To reiterate; are these drugs preventing cellular autophagy, removing the granules or altering the fundamental ageing pattern of the cells? It is a matter of some urgency to establish which is the primary effect using critical and quantitative methods.

SUMMARY

Observations on the age-related volume composition of lipofuscin and hormone-containing organelles in the neurosecretory neurones of the supraoptic nucleus of the hypothalamo-neurohypophyseal system of the ageing C57BL mouse are presented. There is an age-related accumulation of lipofuscin in these neurones with age and although there are changes in the relative volumes of hormone containing structures as determined by quantitative ultrastructural techniques, these are not necessarily connected with lipofuscin accumulation. Under conditions of osmotic stress, the hormone containing organelles remain at a static level whereas lipofuscin pigment is significantly reduced. Evidence is put forward for a turnover of lipofuscin in these cells, and for the presence of this pigment being an indicator of intracellular damage. It is argued that there is insufficient knowledge available concerning the role of lipofuscin in ageing cells and that under these circumstances, it is a matter of urgency to determine this prior to the irresponsible use of drug treatments to reduce pigment content.

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Vol.
16
1981

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THE DISTRIBUTION OF FETAL MORTALITY IN AGEING C57BL/6J MICE: A STATISTICAL ANALYSIS

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Vol.
16
1981

INTRODUCTION

THE DEAD fetuses of polytocous species are removed by resorption *in utero* since they cannot be evacuated without disturbing the living survivors. The process of resorption involves progressive autolysis and phagocytosis until the products of conception have disappeared. Fetal resorption is common in mice and especially so in middle-aged animals where it is largely responsible for the decline in litter size (Finn, 1962; Talbert, 1971). The uterus in ageing mice clearly is less capable of supporting fetal development to term as shown by transplantation of embryos (Talbert and Krohn, 1966), though there is in addition an important contribution from chromosomally aberrant zygotes (Yamamoto *et al.*, 1973; Gosden, 1973; Fabricant and Schneider, 1978).

In the present study the occurrence of fetal resorption was analysed statistically in a large number of ageing mice. For this purpose it was necessary to regard aberrant zygotes as random "noise" because the causes of death cannot be ascertained for individuals at advanced stages of resorption. A statistical approach to the general problem of fetal resorption was adopted to help us infer whether deaths occur as a result of systemic or local causes and, hence, to construct new hypotheses for experimental testing.

MATERIALS AND METHODS

The animals used in this study were multiparous C57BL/6J mice aged 11–12 months; they were obtained from the Jackson Laboratory, Bar Harbor, ME. Young male C3HeB/FeJ mice were obtained from the same supplier. These animals formed part of a previous study (Holinka *et al.*, 1979). Each female was placed with a male of proven fertility and the day of copulation, designated as day 1 of pregnancy, was identified by the presence of a vaginal plug.

A total of 177 mated animals were allocated randomly to groups which were autopsied on days 8–19 of pregnancy, inclusive. The uterine horns were dissected and examined macroscopically; the numbers and position of living and resorbing implants were recorded as described previously (Holinka *et al.*, 1979). The methods of statistical analysis were based on principles described by Siegel (1956) and Colqhoun (1971).

RESULTS

The total numbers of implantation sites per uterus in 11–12-month-old mice did not vary between days 8 and 19 *post-coitum*. Resorbing implants were seen first on day 10 and they increased on the following three days until they represented approximately one-third of the total numbers (Fig. 1). Between days 13 and 19 of pregnancy there was no significant variation in the number or proportion of resorption sites and survivorship in a horn was independent of that in its partner. The right uterine horn carried an average

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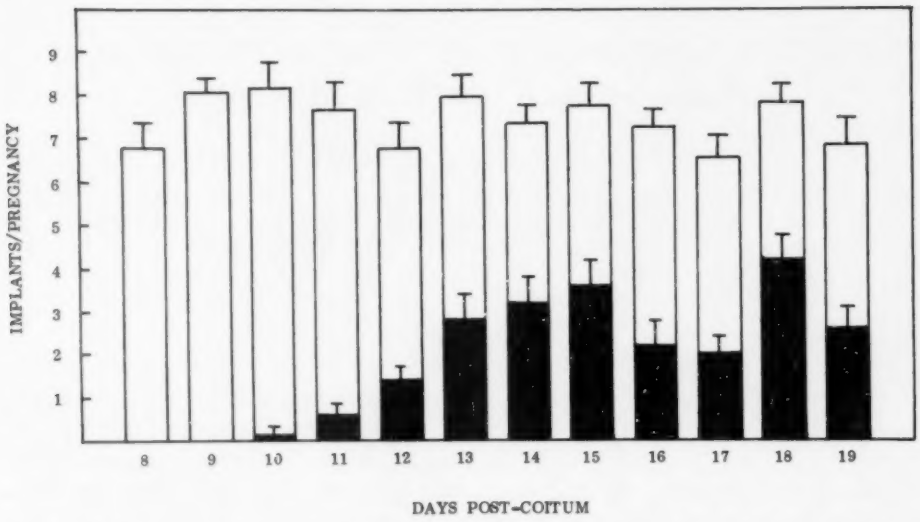

FIG. 1. The height of each bar represents the mean number of live and dead implants per pregnancy in 11–12-month-old C57BL/6J mice. Solid bars indicate the contribution from resorbing implants and vertical lines mark the S.E.M.

of 4.1 implants compared with 3.4 implants in the left horn ($P < 0.01$), a result which closely matches the dextral bias in ovulation number in this strain (Gosden, unpublished observations).

The results obtained from animals examined on days 13–19 were combined for subsequent analyses. This sample comprised 104 animals which were carrying a total of 479 live and 295 dead implants (774 total implants). The animals were then grouped according to the size of their litter but the proportions of resorbing implants did not differ significantly between groups (Table 1).

To examine the question of whether the nine mice with whole litter resorption belonged to a functionally different sub-population the Null hypothesis was adopted that the survival of each fetus was independent and that there was an overall probability of

TABLE 1. ANALYSIS OF EMBRYONIC RESORPTION IN AGEING C57BL/6J MICE WITH SPECIAL REFERENCE TO THE OCCURRENCE OF WHOLE LITTER RESORPTION

	Total number of implants				Resorbing litters		
Litter size	Live	Resorbing	(%)	Total number of litters	Expected number*	(Variance)	Observed number
3	2	1	(33.3)	1	0.0554	0.0523	0
4	13	7	(35.0)	5	0.1055	0.1033	0
5	27	18	(40.0)	9	0.0728	0.0718	1
6	41	49	(54.4)	15	0.0460	0.0458	4
7	85	55	(39.3)	20	0.0234	0.0233	2
8	132	68	(34.0)	25	0.0111	0.0111	1
9	98	55	(35.9)	17	0.0029	0.0029	0
10	58	32	(35.6)	9	0.0006	0.0006	1
11	23	10	(30.3)	3	0.0001	0.0001	0
Total	479	295		104	0.3178	0.3112	9

*Derived from the binomial distribution.

295/774 of being resorbed. The binomial distribution was then used to calculate for each litter size the probability of all fetuses undergoing resorption (Table 1). Hence, the expected number of mice with whole litter resorption was calculated and compared with the observed number. Since the expected number and variance were close, the Poisson distribution was used to estimate the probability of there being nine mice or more with total resorption. This turns out to be extremely small ($p < 10^{-11}$). This indirect test assumes that the risk of resorption is the same for all litters. Variation of this risk could lead to a larger expected number of mice with total resorption without there necessarily being two populations. It would also lead to larger numbers of litters having $(X-1)$ resorbing fetuses than would be expected under the above Null hypothesis where X is the litter size. The expected and observed number of mice with $(X-1)$ resorbers was therefore examined, but here there was close agreement with the Null hypothesis.

Uterine horns were grouped according to the total number of implants they were carrying (a range of 3–7 implants/horn) for an analysis of the occurrence of dead implants in relation to their order along the horn (Cochran's Q test). However, there was no significant heterogeneity ($p > 0.05$).

During an examination of the raw data it was noticed that resorption sites tended to occur adjacent to each other in the same uterine horn. This possibility was evaluated by calculation of the expected number of adjacent resorbing implants and the variance for every horn and comparing it with the observed number. Three adjacent resorbers were counted as two adjacent pairs. Although resorption sites may occur in groups, this tendency did not reach a conventional level of statistical significance ($p > 0.10$).

Vol. 16 1981

DISCUSSION

Two general conclusions stand out from these results. Firstly, animals with whole litter mortality and those with sub-total mortality should in future be considered separately since they appear to comprise distinct sub-populations of ageing animals. The systemic factor(s) responsible for the loss of whole litters cannot yet be identified. Luteal insufficiency can be excluded as an important factor because plasma progresterone levels were not reduced by partial or total loss of conceptuses (Holinka *et al.*, 1979 and unpublished results), a finding that can be attributed to continuing luteotrophic activity by decidual tissue (Pepe and Rothchild, 1974).

Secondly, *prima facie* evidence was obtained that the survivorship of individual fetuses was limited by local factors acting approximately randomly along the uterine wall. In young adult animals the growth potential and vulnerability of fetuses varies in a systematic fashion along the length of the uterine horn and probably reflects differences in the vascular perfusion of the placentas at each point along the arterial arcade (McLaren and Michie, 1960; Trasler, 1960; Beck, 1967; Barr, Jensh and Brent, 1970). In young mice made "superpregnant" by transplantation of large numbers of embryos, fetal mortality during the mid-gestational period increased with crowding above a threshold of eight implants/horn which may be a result of limited uterine surface available for implantation (McLaren and Michie, 1959). Since no comparable effect of fetal position or crowding was seen in ageing animals, other factors are presumably responsible for fetal deaths. Age-related limitations of the pool of available nutrients from the mother do not seem likely since deaths occur before pregnancy exerts its major metabolic draught. Many fetal deaths might result from accidental implantation at unfavourable localities

distributed at random along the uterine horn. At present, there is little indication of the origin or identity of these putative unfavourable sites though they may include areas which provide an inadequate vascular response to implantation and, in multiparous animals, persistent scar tissue (Gosden, 1979).

SUMMARY

Fetal death in 11–12-month-old C57BL/6J mice is first recognized on day 10 *post-coitum* and it increases in incidence over the subsequent three days until more than a third of all fetuses are affected. Thereafter, there is little change in the incidence of resorption sites which apparently persist until term. Analysis of 104 pregnancies between days 13–19 suggested that the loss of individual fetuses is due to local factors operating at random along the uterine wall whereas whole litter resorption results from a systemic factor, though this is not progesterone deficiency.

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THE RELATIONSHIP BETWEEN THE GOMPERTZ CONSTANT AND MAXIMUM POTENTIAL LIFESPAN; ITS RELEVANCE TO THEORIES OF AGING

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Vol.
16
1981

INTRODUCTION

NUMEROUS experiments made during the 1960's suggested that exposure of animals to ionizing radiation resulting in non-specific life shortening. This led to the formulation of the somatic mutation theories of aging (Szilard, 1959; Curtis, 1966). The pros and cons of the theory were amplified and vigorously debated during the following years, but no agreement was reached and it joined the ranks of the many other persuasive hypotheses of aging for which there is quite substantial positive evidence and major objections (reviewed in Comfort, 1979).

The somatic mutation theory has been revived in a modified way in some of the current molecular theories of aging. The "error catastrophe" theory of Orgel (1963, 1970) proposes that senescence is related to the accumulation throughout life of errors in DNA, which interfere with the functioning and replication of the cell. These in turn may lead to somatic mutation and eventually to cell death. The extent and accumulation of DNA damage may be modified in various ways, by the DNA repair capabilities of the cell and by the amount of redundant genetic information in the genome (Hart *et al.*, 1979). There are however, experimental observations which are difficult to reconcile with the "error catastrophe" theory and its modifications (Tice, 1978) and in fact some findings seem to argue directly against them (Morrow and Garner, 1979). Attempts have been made to incorporate hypotheses of somatic mutation and programmed aging into an overall scheme (Kirkwood, 1977; Calow, 1978), but no clear picture had emerged.

In this paper we explore the relationship between two basic aging constants, the Gompertz constant α, and T, the maximum potential lifespan (the maximum age that a species has achieved, usually in a protected environment). In doing so we have a particular objective in mind—to see whether this relationship will give us any further evidence in favour of or against the somatic mutation theory of aging.

METHODS

The Gompertz constant

If the natural logarithm of the mortality rate per 1000 individuals at time t is plotted against time, t (a semi-log plot), a straight line is obtained which has the mathematical form:

$$R_m = R_0 \exp \alpha t,$$

where R_m is mortality rate, R_0 is the value of R_m at time $t = 0$ and α is the Gompertz constant. We can also arrive at a value for α from survival curves, as described below.

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Where we have used the Gompertz plot, we have estimated α only from the portion covering the adult years, and not from the plot of the entire lifespan. This is because it is difficult to derive estimates of natural mortality during the pre-adult years for many species including humans, because mortality rates may then be extremely high. For example, in many marine fishes, only one in ten thousand or more survive the first few weeks of life (Beverton and Holt, 1959). Mortality rates in the nesting stages and first few months of life of many species of birds are high (Bourliere, 1959). Even large species of mammals, such as the buffalo and the impala, have mortality rates of 50% during their first year of life (Spinage, 1972).

The figures which we have for one well-studied species, humans, also illustrates our point. Mildvan and Strehler (1960) have given a range of α for men from 35 nations as 6×10^{-2} to 12×10^{-2} yr. This variability is related to differences in the initial mortality rate. Societies living in poorer environments, where the infant mortality rate is higher, are also characterised by a slower rate of increase of mortality rate. Burnet (1974) has given age-specific mortality curves for the population of the State of Victoria, Australia for the years 1856–1858 and 1965–1967; these give a value for α of 4×10^{-2}, and 9.2×10^{-2}/yr respectively.

Survival curves

A great deal of data is presented in the form of survival curves i.e., the fraction of a species, $\frac{n}{n_0}$ alive at time t (n = the number alive at t, and n_0 is the value of the number at time $t = 0$). Mildvan and Strehler (1960) showed that the surviving fraction of a Gompertian population may be expressed as

$$\frac{n}{n_0} = \exp \frac{R_0}{\alpha} [1 - \exp \alpha t]$$

where n_0 is the initial population at $t = 0$ and n is the surviving population at time t. Estimates of α and R_0 were obtained by the method of iterative non linear least squares for the mortality data for several populations.

Not all survival curves are suitable for this analysis. Thus Kirkwood and Holliday (1979) have presented survival curves for four hypothetical populations. These range from populations in which survival is exponential with constant mortality at all ages, to populations which show the typical parallelogram of survival (Comfort, 1964), characteristic of a protected population, such as humans, laboratory and zoo animals, in which there is a plateau during adult life, followed by a sharp increase in mortality at later ages i.e., individuals show aging. For our analysis we have used only this latter type of survival curve. Between these extremes, Kirkwood and Holliday (1979) have given two survival curves which are characteristic of many feral populations, neither of which show a distinct plateau during the adult years.

Jones (1959) plotted the logarithm of deaths per 1000 individuals in units of 1/1000th of the lifespan against the fraction of the lifespan for three species—humans, with a maximum potential lifespan of 100 yr, the mouse, with a lifespan of 1000 days and the housefly, with a lifespan of 1000 h. He found that these gave three straight lines, all having the same slope. In units of the maximum potential lifespan, T, therefore, the Gompertz constant, α, is the same for all three species, or αT is a constant. We have tabulated the information from the literature for the values of α and T, and have calculated the product, αT, in order to see whether for a wide range of species, αT will be approximately constant, as Jones' work would suggest.

Sources of variability

We anticipated that the relationship which we would derive could only, at best, be an approximate one. One source of variability is in the Gompertz plot itself (which we have discussed), where there are uncertainties due to the high mortality rates during the juvenile years. Part of the variability is also due to the recorded maximum lifespans which may not be accurate—the lack of sound comparative data has been discussed by Comfort (1964). Sacher (1959) considers that the values for maximum potential lifespan, T, are acceptable if they vary within a factor of 2. Thus if we take the variation in α for humans, 3 and a variability of 2 for T, together these give the product αT a range of 6-fold.

RESULTS AND DISCUSSION

We show in Fig. 1 and Table 1 that for 25 animals ranging from invertebrates to humans, the product of αT lies between 2 and 14. We consider that this range is not unreasonable considering the uncertainties inherent in the values of α and T which are the bases of our calculations.

Our purpose in this analysis was to see whether we might throw any further light upon the validity of the somatic mutation theory of aging. Szilard (1959) and Failla (1960), two of the main proponents of the somatic mutation theory, calculated the rate of mutation in human cells and the resultant cell loss throughout the lifespan and from their equations arrived at a figure for longevity that was very close to the potential lifespan of man. Comfort (1964) has discussed these calculations in some detail and has

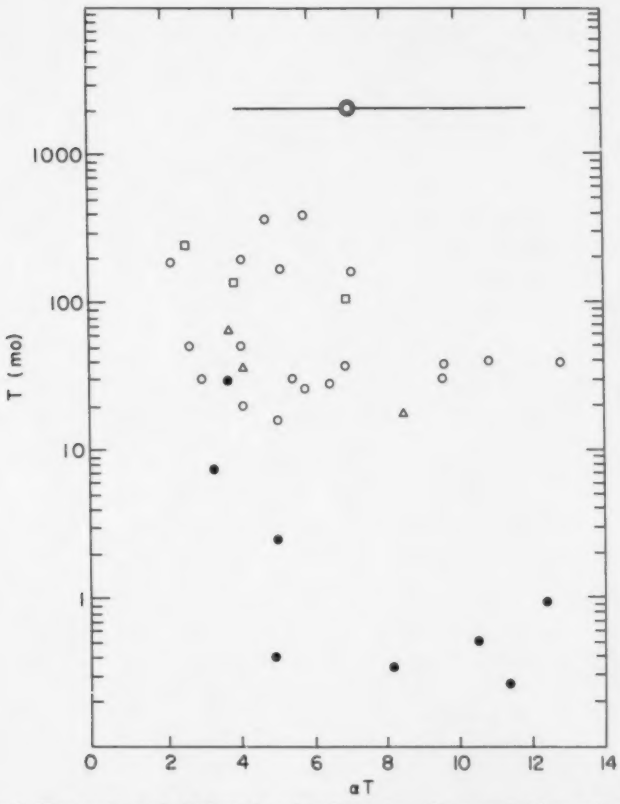

FIG. 1. The product of αT plotted against T (expressed in months) for various species.
0, Mammals; Δ, fish; □, birds; •, invertebrates and ◯, humans.

pointed out that a serious drawback in their hypothesis is that they failed to take the question of cell replacement into account.

In our analysis we have used examples from animals which show considerable differences in their patterns of growth, though all exhibit the "parallelogram of survival". In most birds and mammals, growth virtually ceases at the onset of sexual maturity; on the other hand, fish continue to grow throughout their lifespan although growth rate may decline with age. The list also includes rotifers which have a constant cell number. If somatic mutation and loss of cells is important in aging, we might have expected that animals with different growth patterns might have different αT values; we are encouraged by the lack of any distinctions along these lines. In this context, it is interesting to note that the aging of individual organs is approximately linear (Kohn, 1978), and is not exponential as would be the case if aging were due to somatic mutation.

We set out to see whether the relationship αT would give us any further evidence in favour of or against the somatic mutation theory of aging. Although our data are limited, they do cover a wide range of animals, and so would seem to argue against the theory. This might then lend more weight to alternate theories of aging which are based upon the rate of living, or metabolic rate. Sacher (1959) has given a formula for T which depends mainly on metabolic rate,

$$T \sim \frac{1}{m^{1/4}}.$$

TABLE 1. THE CALCULATED VALUE OF αT FOR VARIOUS SPECIES

Specific name	Common name	α	T	αT	Comments	References
Invertebrates						
Floscularia conifera	Sessile rotifer	$0.78d^{-1}$	10.5d	8.2		Deevey (1947)
Lecane inermis	Rotifer	$0.44d^{-1}$	28d	12.3	(mictic)	Comfort (1964)
		$0.70d^{-1}$	15d	10.5	(amictic)	Comfort (1964)
		$1.41d^{-1}$	8d	11.3		Comfort (1964)
Balanus balanoides	Common barnacle	$0.216m^{-1}$	30m	3.7		Deevey (1947)
Lumnea columella	Pulmonate	$0.015d^{-1}$	225d	3.3		Comfort (1979)
Fumea	Moth	$0.42d^{-1}$	12d	5.05		Comfort (1979)
Drosophilia melanogaster	Fruit fly	$0.66d^{-1}$	76d	2.53		Failla (1960)
Vertebrates						
Lebistes	Guppy	$0.00189d^{-1}$	2000d	3.8		Comfort (1979)
Cynolebias waterstoffi	Bony fish	$0.117m^{-1}$	35m	4.1	15–16°C	Comfort (1979)
Cynolebias waterstoffi	Bony fish	$0.93m^{-1}$	16m	14.9	20–22°C	Comfort (1979)
Cynolebias adloffi	Bony fish	$0.47m^{-1}$	18m	8.5	20–22°C	Comfort (1979)
Maleagus gallopavo silvestris	Brush turkey	$0.26m^{-1}$	150m	3.9		Comfort (1979)
Ardea cinerea	Night heron	$0.11m^{-1}$	250m	2.6		Comfort (1979)
Larus argentatus	Herring gull	$0.735y^{-1}$	9.5y	6.9		Deevey (1947)
Canis familiaris	Cocker spaniel	$0.37y^{-1}$	14y	5.2		Comfort (1979)
Canis familiaris	Pekinese	$0.112y^{-1}$	20y	2.2		Comfort (1979)
Equus caballus	Arabian mare	$0.16y^{-1}$	30y	4.8		Comfort (1979)
Equus caballus	Thoroughbred mare	$0.18y^{-1}$	32y	5.8		Comfort (1979)
Ovis musinam	Mouflon sheep	$0.0205m^{-1}$	200m	4.1		Comfort (1957)
Ovis d. dalli	Dall sheep	$0.525y^{-1}$	13.5y	7.1		Comfort (1957)
Ovis aries	Merino stud	$0.032m^{-1}$	160m	5.1		Comfort (1957)
Microtus orca densis	Orkney vole	$0.24w^{-1}$	125w	3.0		Comfort (1957)
Gerbillus gerbillus	Gerbil	$0.0205w^{-1}$	200w	4.1	♀	Comfort (1979)
Gerbillus gerbillus	Gerbil	$0.0135w^{-1}$	200w	2.7	♂	Comfort (1979)
Mesocricetus auratus	Hamster	$0.32m^{-1}$	30m	9.6		Comfort (1979)
Mesocricetus auratus	Hamster	$0.336m^{-1}$	38m	12.8		Comfort (1979)
Rattus rattus	Rat	$0.0065d^{-1}$	1100d	6.9		Failla (1960)
Rattus rattus	Rat	$0.0049d^{-1}$	1150d	9.6	♀	Comfort (1979)
Rattus rattus	Rat	$0.00727d^{-1}$	900d	6.5	♂	Comfort (1979)
Mus muscaris	Mouse	$0.0075d^{-1}$	850d	6.5		Failla (1960)
Mus muscaris	Mouse	$0.636w^{-1}$	163w	10.4		Comfort (1979)
Homo sapiens	Man	$0.04y^{-1}$ to $0.12y^{-1}$	100y	4–12		Cutler (1979) Burnet (1974) Mildvan and Strehler (1960)

d = days; w = weeks; m = months; y = years

In this paper we have shown that $\alpha T \sim$ constant. We, therefore, suggest that $\alpha \sim m^{1/4}$ and that hence a major factor affecting α is metabolic rate. Indeed there is evidence which suggests that aging is a result of damage due to the production of free radicals in the cell during respiration (Liebovitz and Siegel, 1980) and that longer lived species have a better degree of protection against the debilitative products of oxygen metabolism (Tolmasoff *et al.*, 1980).

SUMMARY

A correlation between the two aging parameters, the Gompertz constant, α and the maximum potential lifespan T is obtained. The product of α and T lies between 2 and

12 for mammals which have ceased to grow in their adult years, fish which continue to grow throughout life and rotifers, which have a constant number of cells. Since T is related to the metabolic rate, m, as $m^{-\frac{1}{4}}$, then α must be related to the metabolic rate as $m^{+\frac{1}{4}}$. It is suggested that somatic mutations play a lesser role than metabolism in aging.

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Vol. 16 1981

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SKIN TEMPERATURE OF BEDSORE AREAS IN THE AGED

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Vol. 16 1981

INTRODUCTION

BEDSORES, pressure sores, or decubitus ulcers, as they may sometimes be termed, tend to occur in certain special areas. The common sites include the sacrum, the great trochanter, the external malleolus and the heel. The pathological process concerned is basically an ischaemic necrosis complicated by external pressure. Hence, the state of local blood supply may be a critical factor. It was, therefore, decided to take the skin temperature at the sites mentioned and to compare this with the readings found in nearby areas in groups of elderly subjects.

MATERIALS AND METHODS

The subjects investigated comprised 50 females aged between 65 and 90 yr old (Table 1). They were all convalescent in-patients at Queen's Hospital, Croydon. None had any evidence of ischaemic lesions; all were active to a moderate degree. Temperatures were always taken between 10 and 11 a.m. with an electric thermometer, made by Light of Brighton, which was accurate to within ± 0.2°C. This instrument reached a steady reading within a few seconds of contact with the skin.

TABLE 1. AGE OF SUBJECTS

Age range	Number
60–69	3
70–79	14
80–89	30
90–99	3
Total subjects	50
Mean age	81 yr
S.D. of age	5.8

The group used for comparison comprised 103 females aged between 61 and 100 yr, who had been investigated in a research project designed to ascertain skin temperature levels at 11 different points on the body, which included groins, ankles and great toes among other sites.

RESULTS

The temperature readings over the body of the sacrum ranged from 33.4 to 36.0°C. This gave a range of 2.6°C and a mean figure of 34.96°C. Over the right great trochanter, the range varied from 28.2 to 35.2°C. The range was 7.0° and the mean was 33.05°C.

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The left great trochanter gave slightly different figures, with a range between 31.0 and 35.0°C, amounting to only 4.0° with a mean of 33.22°C. As might be expected, the readings over the malleoli were lower. The right external malleolus temperature varied from 22.8 to 34.6°C, a range of 11.8° and a mean of 30.16°C. The corresponding figures for the left external malleolus were from 22.0 to 34.6°C. This amounted to 12.6° in range, with a mean of 30.10°C. When the heels were examined, the range fell between 21.4 and 34.8°C on the right and between 21.2 and 34.4°C on the left, an ambit of 13.4° on the right and 13.2°C on the left. The respective mean figures were 30.01° on the right and 29.82°C on the left (Table 2).

TABLE 2. TEMPERATURE RANGE AT BEDSORE SITES (°C)

Site	Minimum/ maximum	Range	Mean	S.D.	S.E. of mean
Sacrum	33.4–36.0	(2.6)	34.96	0.61	0.09
Right great trochanter	28.2–35.2	(7.0)	33.05	1.45	0.21
Left great trochanter	31.0–35.0	(4.0)	33.22	1.06	0.15
Right external malleolus	22.8–34.6	(11.8)	30.16	2.46	0.35
Left external malleolus	22.0–34.6	(12.6)	30.10	2.56	0.36
Right heel	21.4–34.8	(13.4)	30.01	2.91	0.41
Left heel	21.2–34.4	(13.2)	29.82	3.08	0.44

Statistical comment

Differences between right and left side readings at great trochanter, external malleolus and heel are not significant. The spread, or standard deviation (S.D.), increases from sacrum to heel. This reflects changes in arterial circulation towards the limb extremities. Similarly, the mean temperature also falls from 34.96°C over the sacrum to 29.82°C on the heel.

Correlations between right and left side readings are large (0.82, $p < 0.001$) for heel and external malleolus, but rather low for great trochanter (0.42, $p < 0.01$). Since the mean differences for these three sites are not significantly different from zero, but the S.D.s of right and left great trochanters' temperature are significantly different, ($F_{49/49} = 1{,}88$, $p < 0.05$), it is possible that left great trochanter temperature tends to be more constant (around 33.2°C) than the right great trochanter. The high correlations for right and left external malleoli and heels suggests that causes of temperature differences between patients' feet due to circulation are more significant than left-to-right foot differences within each subject.

The correlations between sacrum and great trochanter or foot temperatures are not very large, suggesting that sacral readings give little indication of temperatures in great trochanters, or feet. The general pattern of temperatures in sacrum, great trochanter and foot shows a significant ($p < 0.001$) decrease from sacrum to heel. Differences between right and left sides are insignificant, while right and left foot readings show more correlation than right and left great trochanter temperatures (Tables 2 and 3).

Comparisons with adjacent areas

A previous investigation into skin temperatures at various sites among 103 elderly females gave a basis for comparison with the findings in the present research. When the right great trochanter was compared with the right groin, the former had a maximum reading of 35.2° as against 36.8°C in the fold of the groin. The left great trochanter had

TABLE 3. CORRELATION COEFFICIENTS OF BEDSORE SITES

Site	Correlation coefficient	Value that correlation coefficient is zero
Rt/Lt great trochanter	0.42	< 0.01
Rt/Lt external malleolus	0.82	< 0.001
Rt/Lt heel	0.82	< 0.001
Rt great trochanter/external malleolus	0.46	< 0.001
Rt external malleolus/heel	0.72	< 0.001
Rt great trochanter/heel	0.32	< 0.05
Rt great trochanter/sacrum	0.24	0.09
Rt external malleolus/sacrum	0.22	0.13
Rt heel/sacrum	0.17	0.26
Lt great trochanter/external malleolus	0.17	0.20
Lt external malleolus/heel	0.77	< 0.001
Lt great trochanter/heel	0.21	0.15
Lt great trochanter/sacrum	0.19	0.20
Lt external malleolus/sacrum	0.21	0.15
Lt heel/sacrum	0.19	0.20

There is no significant correlation of site temperature with age.

Vol. 16 1981

a highest figure of 35.0°C while the groin showed 37.8°C. As far as the ankles were concerned, the right external malleolus had a maximum level of 34.6°C, compared with a figure of 36.4°C at the front of the joint. On the left, the external malleolus and the front of the ankle showed exactly the same readings as the right. When the heels were compared with the great toes, the highest reading on the right heel was 34.8°C as against 38.4°C at the great toe. The left heel showed a maximal figure of 34.4°C, while the toe had a highest figure of 36.4°C. It will be seen, therefore, that the maximum temperature of the sites liable to have bedsores was always less than that of points only a few cm away.

When minimal temperature readings were compared in the same way, a somewhat different picture emerged, as may be seen in Tables 4 and 5. The lowest reading at the

TABLE 4. AGE OF COMPARISON GROUP

Age range	Number
60–69	8
70–79	34
80–89	47
90–99	15
100+	1
Total number	103

TABLE 5. TEMPERATURE RANGE IN COMPARATIVE SITES (°C)

Site	Minimum	Maximum	Range
Right groin	33.0	36.8	3.8
Left groin	32.4	37.8	5.4
Right ankle	22.2	36.4	14.2
Left ankle	21.2	36.4	15.2
Right great toe	20.2	38.4	18.2
Left great toe	20.0	36.4	16.4

right great trochanter was 28.2°C, as against 33.0°C in the right groin. The left great trochanter showed a minimum figure of 31.0°C, compared with 32.4°C in the groin. At the ankle, the right external malleolus had its low figure of 22.8°C. But the front of the right ankle could be lower still, 22.2°C. On the left side, the external malleolus had a minimum of 22.0°C, while the front of the ankle showed a low level of 21.2°C. The right heel had a minimum of 21.4°C, while the right great toe might be as cold as 20.2°C. The left heel showed its lowest reading at 21.2°C. The left great toe had its minimum figure at 20.0°C. Thus we can see that while both great trochanters had lower figures than the groins, the other sites did not. These findings indicate that the range of skin temperature was less in the bedsore areas than at adjacent sites, except for the right great trochanter as compared with the right groin.

It was difficult to find any area comparable with the sacrum. The nearest kind of level, anatomically, was the abdominal wall. Here the maximum temperature registered between the xiphisternum and the umbilicus was 36.8°C, while the minimum was 33.0°C. This ambit of 3.8°C has to be considered with the sacral range of 33.4–36.0°C, which equals a mere 2.6°C. Since, however, the comparison was made between two different groups of subjects, it should be necessary to allow for a certain amount of error on this account.

DISCUSSION

In Barton's thermographic studies of bedsores (1973), he reports figures between 32.6 and 34.5°C in different parts of an indolent sacral bedsore occurring in an elderly patient who died shortly afterwards. He also mentions figures of 32.5 and 37.0°C in another sacral sore of "hyperactive" type which was healing. It is interesting to compare these temperatures with the range of 33.4–36.0°C in the present series, taken on unbroken skin. The lowest figure occurring in an actual ulcer is definitely lower than the minimum in the subjects under review.

Zones subject to bedsores in the series examined have a maximum temperature lower than that of nearby areas, as found in a previous investigation (Howell, 1980). As regards minimum readings, however, these are not always below those found in the areas of comparison. There seems to be an overlap in certain instances.

On the whole, the present research supports the concept that sites liable to bedsores have a less efficient circulation than areas which are anatomically adjacent. It provides standards by which normal skin temperature can be judged. Any diminution may be associated with tissue necrosis and ulceration, if Barton's figures are typical.

SUMMARY

The skin temperature has been taken at four sites where bedsores commonly occur; sacrum, great trochanters, external malleoli and heels, among fifty elderly women. These have been compared with readings in adjacent sites at which skin temperature was measured in a previous investigation. Maximum readings at bedsore areas were lower than those in the zones of comparison. Minimum readings had an overlap between bedsore areas and areas of comparison. There was no significant correlation of temperature at bedsore sites and ages, nor between right and left sides of the body. Present results can provide a standard by which to judge cases in danger of developing bedsores.

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AGEING CHANGES IN PORTON RATS WITH SPONTANEOUSLY ARISING PITUITARY TUMORS

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Vol.
16
1981

INTRODUCTION

AGED rodents frequently exhibit many types of pathologic changes thereby often making difficult the distinction between tissue changes arising spontaneously and those induced experimentally. However, this distinction should become easier as more information becomes available on the strain specific incidences of spontaneously arising lesions (Cohen *et al.*, 1978). Although the pathogenesis remains obscure, considerable data has now been published on spontaneously arising kidney lesions in aged rats and similar data on strain specific spontaneously occurring tumor incidences is also gradually accumulating.

The kidney lesions reported in aged rodents have been variously specified as glomerular sclerosis (Couser and Stilmant, 1975; Elema and Arends, 1975) chronic nephropathy (Coleman *et al.*, 1977) chronic nephritis (Snell, 1967) and chronic nephrosis and glomerulonephritis (Simms and Berg, 1957). Though given different names by different investigators, it seems that in general, these kidney lesions do arise spontaneously and they occur more frequently and more severely among older rats. These renal changes have been noted in several inbred rat strains and among those housed conventionally or maintained specific pathogen free (Coleman *et al.*, 1977). In addition Snell (1967) reported that females of several strains were less susceptible to these changes than were males.

Just as with renal changes, tumors too occur more frequently among older rats but there is a great deal of strain specific variation in incidence. One commonly reported rat tumor is a pituitary adenoma (Simms and Berg, 1957) also designated chromophobe adenoma (Kim *et al.*, 1960), which can reach a 60 per cent incidence in aged male Yale strain rats (Saxton and Graham, 1944). In one case this tumor-type has been linked with high serum prolactin levels and mammo-tropic activity (Ito *et al.*, 1972) and Coleman *et al.*, (1977) reported fatty changes of the inner adrenal cortex of Fischer 344 male rats bearing chromophobe adenomas of the pituitary. In another study the incidence of these tumors has been shown to be influenced by dietary protein levels and caloric intake (Ross *et al.*, 1970).

The complex inter-relationships between the hypothalamic pituitary axis and ageing rates in mammals has been investigated in several ways (Everitt and Burgess, 1976). In many studies ageing rates in rats have been measured by recording the breaking time of stressed tail tendons, and in one study, the removal of the pituitary gland from rats at 46–65 days of age has been shown to retard this measurement of the collagen ageing process (Delbridge and Everitt, 1972). The possibility of accelerating ageing rates by increased tumor-related hypothalamic and/or pituitary activity however has not been investigated.

In a life-span feeding trial in rats we had an opportunity to observe many spontaneously arising tissue changes and to test the hypothesis that animals bearing spontaneously arising pituitary tumors might have more advanced age-related changes than their tumor-free cohorts.

METHODS AND PROCEDURES

Thirty outbred Porton rats were obtained specific pathogen free, thereafter housed conventionally and placed on one of three diets for four weeks prior to mating. The diets differed markedly in quantity and quality of their dietary fats. The diets were designated: stock, a low-fat, conventional cereal based cube, control, a high-fat, human-type diet with 2.1% polyunsaturated fat and poly, a high-fat human-type diet with 21% polyunsaturated fat. The latter two diets were freeze-dried, vacuum sealed and kept frozen until used.

The parent animals and some of their offspring were kept on these diets throughout their life-span but other siblings were killed at 3 months, 1 yr and 2.1 yr for serum biochemical and histological studies. Although differing in fat quantity and quality, the diets were fed at levels which equalised growth rates between diets, hopefully minimising caloric dependent growth rate differences. These results and further details of the diet are reported elsewhere (Horn *et al.*, 1979).

All animals, whether killed or dead from spontaneous disease processes, were autopsied, tissues fixed in formal-saline or Bouins and paraffin sections of tissues were routinely stained with hematoxylin and eosin. Longitudinal kidney sections were scored by two people independently using the scheme published by Coleman *et al.* (1977) in an attempt to quantitate the nephropathy.

Collagen tail tendon fibres were excised and kept moist in phosphate buffered saline until used. Breaking time in 7 M urea at 40°C. was recorded using a 2 g weight tied to the end of the tendons. Further details of this and other ageing parameters studied on these rats are published elsewhere (Horn *et al.*, 1981).

RESULTS

Kidney tissues from animals killed at 3 months, 1 yr and 2.1 yr provided the background data on spontaneously arising renal changes and there did not seem to be any change which was associated with a specific diet. Although 3 months and 1-yr old animals had few kidney changes, as expected, the whole organ weights and glomerular size increased with increasing age. By 2.1 years of age we routinely noted a thickening of the basement membranes in the glomeruli and in some cortical tubules (Fig. 1). These changes were scored as equivalent to Coleman's grade 1 chronic nephropathy (Coleman *et al.*, 1977) and the kidney sections of 36 per cent of animals killed for biochemical studies at 2.1 yr of age were given that score. In addition 52 per cent of 2.1-year old animals also had scattered hyline casts and occasional dilated tubules; the presence of these changes plus some atrophic tubular epithelium were considered as equivalent to Coleman's grade 2. The remaining 12 per cent of the animals killed at 2.1 yr were scored grade 3 chronic nephropathy because dilated tubular epithelium had become a prominent feature usually accompanied by occasional glomerular sclerosis.

Only the 25 animals from all three diets that were tumor free when killed at 2.1 yr of age were included in this scoring scheme and they formed the basis of the "expected" age-related renal changes for this rat strain under our conditions (Fig. 2).

Other spontaneously arising kidney changes noted in the Porton strain were: a < 2 per cent incidence of congenital renal asymmetry with one organ one-third to one-half normal size accompanied by a compensatory hypertrophy of the opposite kidney, a < 2 per cent incidence of bilateral renal and bladder calculi and a < 1 per cent incidence of acute pylonephritis ascending from prostatic abscesses. As with the age associated renal changes, none of these conditions seemed to be diet specific.

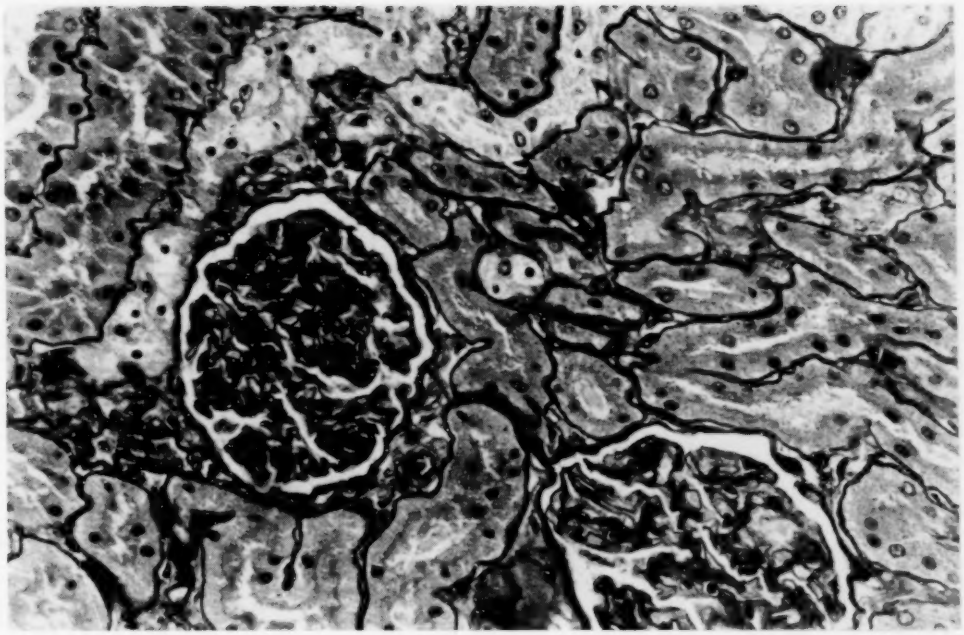

FIG. 1. Kidney from 2.1-yr old Porton rat which received a 1+ score for age-related kidney changes (PAS stain).

Vol.
16
1981

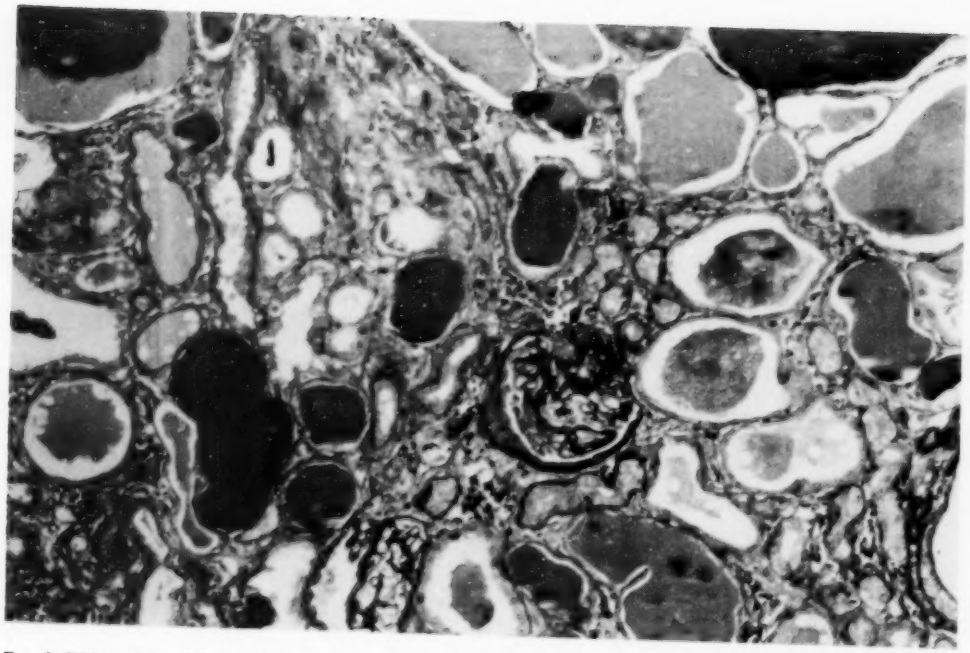

FIG. 3. Kidney from 1.2-yr old Porton rat which died of a pituitary tumor. This kidney received a score of 4+ (PAS stain).

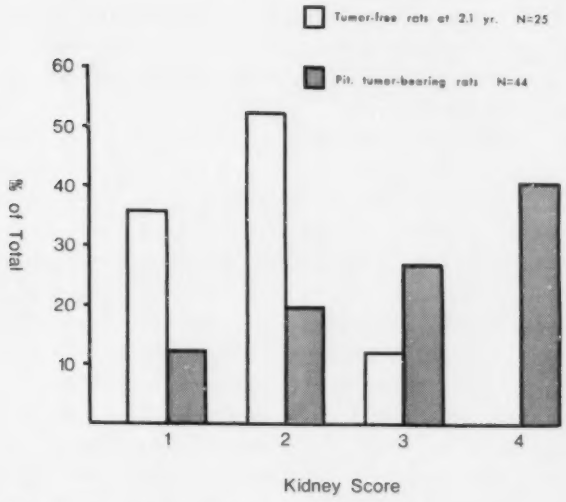

FIG. 2. Age-related kidney changes (Coleman's scores of 1+ to 4+) among Porton rats with pituitary tumor and their tumor free cohorts.

Vol. 16 1981

In this life-span feeding trial, pituitary tumors were the most common tumor type (Table 1) and they varied in size from microscopic to over 1 cm in dia. The latter were frequently cystic, blood filled and caused compression lesions to adjacent overlying neural tissue. As with the kidney changes, there were no differences in incidence or severity between diets but overall pituitary tumors were more common among females. Some animals with pituitary tumors exhibited clinical signs of weight loss and incoordination prior to death while others were asymptomatic and < 5 per cent of the tumors were overtly mammotropic. Not all of these pituitary tumors were fatal and some animals had other tumors in addition to pituitary tumors. Among animals on the high fat diets the apparent lower incidence for offspring vs parent animals to some extent reflects the higher incidence of fatal pulmonary disease among the offspring at about 2 yr of age (Horn *et al.*, 1979).

TABLE 1. INCIDENCE OF FATAL AND NON-FATAL PITUITARY ADENOMAS AMONG PORTON RATS ON A LIFE-SPAN FEEDING TRIAL

	No. of animals with pituitary tumors / No. of animals "at risk"		
	Poly diet	Control diet	Stock diet
Life-span animals	7/50 (14%)	9/45 (20%)	19/52 (36%)
Parent animals	11/20 (55%)	12/20 (60%)	6/20 (30%)
Totals	10/70 (26%)	21/65 (32%)	25/72 (35%)
Female/male ratio	15 : 3	14 : 7	16 : 9
Average age (yr) at death	2.4	2.3	2.5

The most common histologic feature of these pituitary tumors was a cell-type with little discernible cytoplasm: the large nucleus showed marked margination of the nuclear chromatin. Few mitotic figures were noted and differential staining using Cameron and Steel's method to further elucidate cell-type was not helpful (Pearse, 1972). As previously

mentioned these tumors, especially the larger ones, frequently had blood filled sinusoid cysts. Other tumor types were seen at much lower frequencies than the pituitary tumors (Horn *et al.*, 1979) and among the 207 animals autopsied there was only one other brain tumor which was not a pituitary tumor.

One of the first animals to die of a pituitary tumor (at 15 months of age) also had extensive renal pathology (Fig. 3). This lead us to hypothesize that in general, pituitary tumors might accelerate this age related change. Thereafter, kidney sections from animals bearing fatal or non-fatal pituitary tumors were prepared, examined and then scored in the same way as were the kidneys from animals killed at 3 months, 1 yr and 2.1 yr of age. Using this method, 12 per cent were scored at 1+, 20 per cent as 2+, 27 per cent as 3+ and 41 per cent as 4+. When compared to tumor-free animals at 2.1 yr of age, the animals with pituitary tumors had more severe chronic nephropathy (Fig. 2). An observation further supported by the fact that no tumor-free animals were given 4+ scores. Tumor bearing animals with kidney scores of 1+ were not necessarily those with the smaller non-fatal tumors but animals 2.5 yr of age and older with fatal pituitary tumors (frequently) 1–1.5 cm dia.) all had extensive chronic nephropathy. These severe kidney changes were so consistently associated with pituitary tumor bearing animals as to become a reliable "diagnostic predictor" at necropsy. Other endocrine tissues showed expected age related histologic changes but none correlated with tumor-bearing.

In a previous publication we reported good correlations between chronological age and the breaking time of stressed tail tendons (Horn *et al.*, 1981). Using this method, the tail-tendons from 28 pituitary tumor bearing animals were similarly tested. When groups of tumor bearing vs tumor-free animals for each diet were tested there was no evidence of differences between breaking times. All pituitary tumor bearing animals were then grouped together regardless of diet and compared to their tumor-free cohorts over 15 months of age. Fifteen months was chosen because this was the youngest age at which a pituitary tumor was diagnosed. Although a trend was apparent which suggests that pituitary tumor bearing animals have longer tail-tendon breaking times, the differences were not statistically significant.

DISCUSSION

In this life-span study we saw an age-related pattern of kidney changes which was similar to that described by Coleman *et al.* (1977) but among tumor-free animals killed at 2.1 yr the severity was markedly less than that reported for the Fischer rats. The majority of their 2.1-yr old animals were scored as having 3+ and 4+ changes whereas 52 per cent of the 2.1-yr old Portons were scored 2+ and none were scored 4+.

Serum urea nitrogen can be used as an indirect measure of kidney function and with this criteria too the Porton rats showed less age-related change than did the Fischer rats. Group mean values from our rats ranged from 13.1 mg/dl for control fed males at 1 yr ($N = 10$) to 25.1 mg/dl for stock diet fed females at 1 yr ($N = 10$). All of the group mean values for 2.1-yr old animals were at least 10 per cent less than this latter figure ($N = 26$) whereas Coleman reported 25.8 mg/dl for male Fisher rats 24–30 months of age.

Further supporting evidence that our tumor-free rats had less spontaneous age-related kidney disease was from a subsidiary study investigating urinary urea nitrogen excretion. Here 44 rats either 3 months or 25 months of age showed no age-related alterations in the pattern of urea excretion.

Although tumor-free Porton rats showed less severe spontaneous kidney disease than did Fischer rats the majority of Porton rats with pituitary tumors had markedly more severe chronic nephropathy than did their tumor-free cohorts. This correlation between pituitary tumors and severe kidney disease suggested to us that perhaps there was some functional tumor activity causing an acceleration of the renal ageing. Although the possibility of an excess of anti-diuretic hormone (ADH) via the posterior pituitary cannot be positively ruled out, our evidence and that of others (Furth *et al.*, 1976) suggests that these heritable pituitary adenomas are tumors of the anterior section of the gland and as such should not be secreting ADH. Whatever the mechanism, the suggestion is consistent with recent findings that hypophysectomy at young ages retards age-related renal disease as assessed by proteinuria (Everitt *et al.*, 1980).

Most gerontologists would accept that in mammals, the hypothalamus and pituitary have a general influence on ageing rates and as previously mentioned, ablation of the pituitary from young rats has been shown to delay collagen ageing as measured by the tail-tendon tension test (Delbridge and Everitt, 1972). We therefore hypothesized that a functionally hyperactive tissue may be at least partially responsible for the accelerated ageing of kidney tissue that we noted. Our tail-tendon collagen data from pituitary tumor bearing animals is only suggestive of such a relationship but we would like to encourage other investigators who have rat colonies with a high incidence of pituitary tumors to examine this question further.

Vol. 16 1981

SUMMARY

In a life-span study investigating effects of dietary fats we found that extensive renal pathology correlated with the presence of pituitary adenomas especially of young ages. We suggested that these tumors were functional and somehow accelerating this age-related tissue pathology. There was no evidence, however, that pituitary tumors altered ageing rates as measured by the breaking time of collagen tail tendons in 7 M urea at 40°C.

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ROLE OF CELL SURFACE MORPHOLOGY ON AGE-RELATED CHANGES IN CONCANAVALIN A-MEDIATED RED BLOOD CELL ADSORPTION CAPACITY OF HUMAN DIPLOID FIBROBLASTS

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Vol.
16
1981

INTRODUCTION

IN A PREVIOUS paper, we have reported that two methods of concanavalin A (Con A)-mediated red blood cell (RBC) adsorption assay can reveal two types of age-related changes in the surface of human diploid fibroblasts (Aizawa *et al.*, 1980*a*). RBC adsorption with the RBC coating method (in which Con A-coated RBCs are adsorbed to fibroblasts) increased continuously from early passages up through the retardation of cell growth. On the other hand, RBC adsorption with the fibroblast coating method (in which RBCs are adsorbed to Con A-coated fibroblasts) only occurred to phase III cells and did not occur to phase II cells. Considering the possible implications of the two types of age-related changes in cell surfaces of human diploid fibroblasts revealed by RBC adsorption (Aizawa *et al.*, 1980*b*; Aizawa, 1980), a clarification of the substantial basis of the age-related changes and in this regard the difference between the results with the RBC and fibroblast coating methods might help in our understanding of the role of cell surface changes on cellular aging in general (Aizawa and Kurimoto, 1979*a, b*; Aizawa *et al.*, 1980*c*).

Lectin-mediated agglutination of cells has been reported to be influenced by cell surface morphology such as microvilli, and increased agglutinavility of transformed cells has been sometimes explained in this term, although the data have been conflicting each other (Collard and Temmink, 1976; Oppenheimer *et al.*, 1977; Porter *et al.*, 1973; Vlodavsky and Sachs, 1977; Willingham and Pastan, 1975). In addition, changes in surface morphology such as increase in microvilli and blebs have been known to occur with aging of human diploid fibroblasts (Crusberg *et al.*, 1979; Johnson, 1979; Wolosewick and Porter, 1976). In the present paper, we have examined the role of cell surface morphology on Con A-mediated RBC adsorption with scanning and transmission electron microscopy.

MATERIALS AND METHODS

Human diploid fibroblasts (TIG-1) from the lung of a female fetus were cultured in MEM supplemented with nonessential amino acids (GIBCO, G-77) and with 10% fetal bovine serum (GIBCO, I-76) as previously described (Aizawa *et al.*, 1980*a*). RBC adsorption assay was also performed as previously described (Aizawa *et al.*, 1980*a*). For scanning electron microscopy (SEM), cells were plated on 10.5 × 22 mm plastic cover slips (Thermanox). After RBC adsorption, RBC-adsorbed fibroblasts were fixed in 0.1% glutaraldehyde and treated by a routine schedule for SEM; post osmification and single gold coating with an Ion Coater, IB-2 (Eiko Engineering, Japan) after critical point drying. Cells were examined in a JXM-50A scanning microscope. For transmission electron microscopy, cells were plated on 35 mm plastic dishes (Lux). After RBC adsorption, cells were processed as previously described (Kaiho and Sato, 1978) and examined in a JEM-100C electron microscope.

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RESULTS

In contrast to suspended round cells, monolayer-cultured cells are not uniform in their surfaces. Distribution and movement of Con A receptors can be questioned to be different from part to part of cell surfaces (Vasiliev *et al.*, 1976). As shown in Figs. 1–3, however, Con A-mediated RBC adsorption occurred to all parts of cell surfaces including thin processes (Fig. 1), thick processes (Fig. 2), lamelloplasm (Fig. 3), endoplasm (Fig. 3), cell periphery, cell center and so on. Thus, there was no special loci for RBC adsorption. This was the case regardless of the coating method and of population doubling levels (PDLs) of cells, although the amount of RBC adsorption was lower with the fibroblast coating method and with lower PDLs of cells.

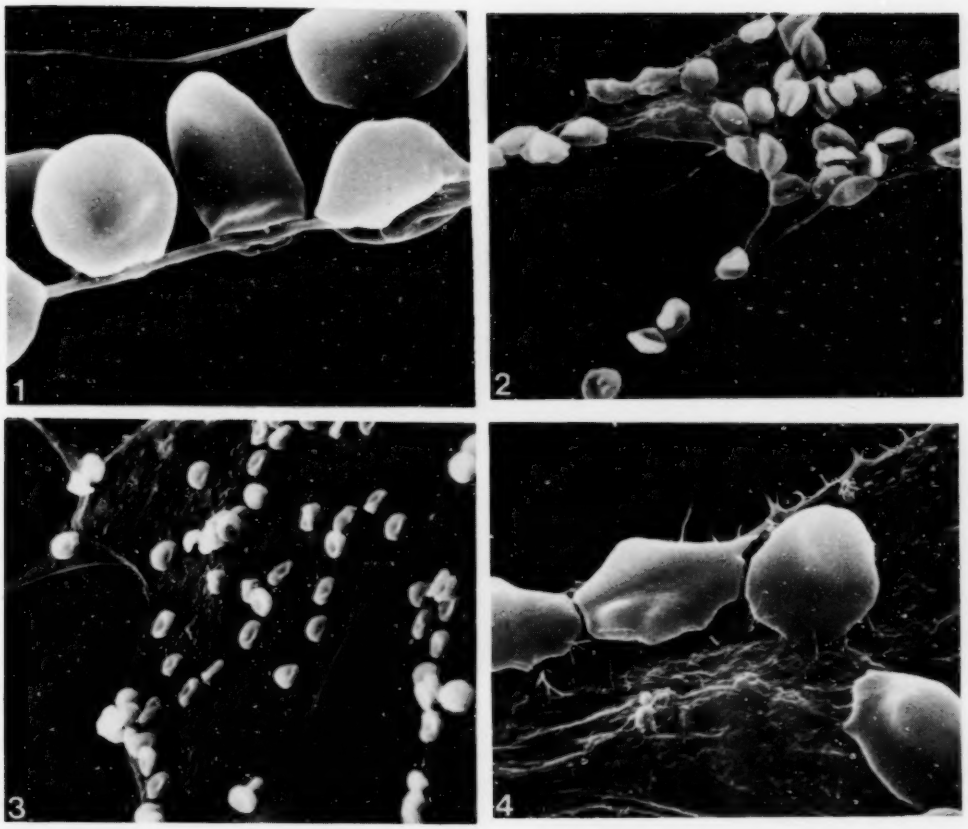
The age-related increase in microvilli has been known (Johnson, 1979), and the correlation between increase in Con A-mediated agglutination of cells and in microvilli has been also reported (Willingham and Pastan, 1975). Sometimes the participation of microvilli was surely obvious also in RBC adsorption (Fig. 4), but it was not necessarily the case. The adsorption occurred to either area that have smooth surfaces (Fig. 3) or numerous microvilli (Fig. 4). Rather than microvilli, the flat areas of opposed membrane were also more frequently observed with the transmission electron microscopy at points of RBC-fibroblast contact (Figs. 5 and 6), regardless of the coating method and PDLs of cells.

Although changes in the surface morphology such as microvilli have been reported by treatment of lymphocytes with Con A (Temmink *et al.*, 1976), no obvious change in cell surface morphology was found by the treatment of human diploid fibroblasts with Con A, as it was also the case for some other types of cells (Willingham and Pastan, 1975; Collard and Temmink, 1976). However, the deformation of adsorbed RBCs was frequently observed along the intricated surfaces of fibroblasts (Figs. 7 and 8), and this was pronounced when RBC-adsorbed fibroblasts were stood for longer time before fixation. As previously reported, adsorbed RBCs were gathered into clumps on surfaces when RBC-adsorbed fibroblasts were cultured (Aizawa and Kurimoto, 1979*c*). Fixation of Con A-uncoated counterpart cells (RBCs in the fibroblast coating method and fibroblasts in the RBC coating method) decreased the RBC adsorption (Aizawa and Kurimoto, 1979*a*). The deformability of cell surfaces might have some participation in RBC adsorption.

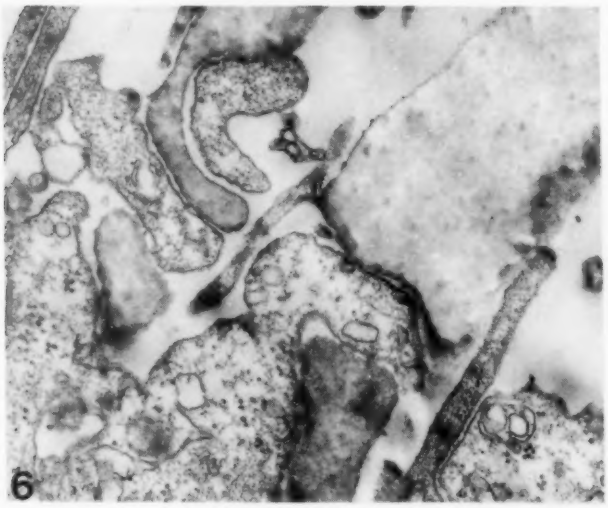
DISCUSSION

Involvement of microvilli in Con A-mediated agglutination was proposed by Porter *et al.* (1973) and received an experimental support from the work of Willingham and Pastan (1975). Willingham and Pastan found that transformed, agglutinable cells had more microvilli than normal, non-agglutinable cells. In addition, treatment with dibutyryl cAMP reduced the number of microvilli and concomitantly decreased the agglutinability of the transformed cells. Thin sections of agglutinated cells showed a forest of interdigitating microvilli at the points of cell attachment. On the other hand, Collard and Temmink (1976) reported non-agglutinable 3T3 cells had more microvilli than agglutinable SV-40 3T3 cells. In addition, there is a report that transmission electron micrographs of agglutinated cells has shown flat areas of opposed membrane (Nicolson, 1972). More recently, Ukena and Karnovsky (1977) reported that the morphology of cells, microvilli, could not explain the variable agglutinability of eight cell lines examined. In an agreement with their conclusion on Con A-mediated agglutination of cells, our present result suggests that the cell surface morphology also cannot explain the age-related changes in RBC adsorption and the difference between the results with the

Vol.
16
1981

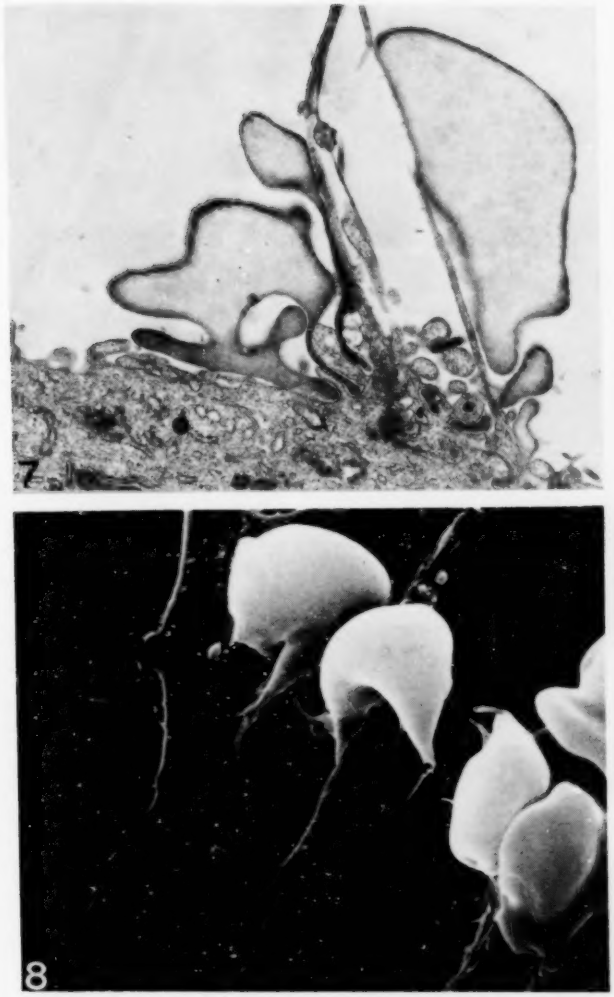


FIGS. 1–4. SEM photographs of Con A-mediated RBC adsorption to human diploid fibroblasts. Fig. 1, RBC adsorption to PDL 61 cells with the RBC coating method (×4100); Fig. 2, to PDL 61 cells with the RBC coating method (×1100); Fig. 3, to PDL 63 cells with the fibroblast coating method (×800); Fig. 4, to PDL 61 cells with the RBC coating method (×4100).



FIGS. 5 and 6. Tem photographs of Con A-mediated RBC adsorption to human diploid fibroblasts. Fig. 5, RBC adsorption to PDL 46 cells with the RBC coating method (×4600); Fig. 6, to PDL 46 cells with the RBC coating method (×68,400).

Vol.
16
1981



FIGS. 7 and 8. Deformation of adsorbed RBCs. Fig. 7, a TEM photograph of RBCs adsorbed to PDL 46 cells with the RBC coating method (×9000): Fig. 8, SEM photograph of RBCs adsorbed to PDL 61 cells with the RBC coating method (×4400).

two coating methods. The result is also supported by our previous results that drugs such as Ca ionophore, local anaesthetics and dibutyryl cAMP, which have been known to affect microvilli formation (Ukena and Karnovsky, 1977; Vlodavsky and Sachs, 1977; Willingham and Pastan, 1975), did not have any pronounced effects on RBC adsorption (Aizawa and Kurimoto, 1979*b*).

SUMMARY

We previously described two types of age-related cell surface changes which could be revealed in human diploid fibroblasts by concanavalin A-mediated red blood cell (RBC) adsorption with the RBC and fibroblast coating methods. The role of cell surface morphology on these age-related cell surface phenomena and the difference between the results with the two coating methods was examined with scanning and transmission electron microscopy. RBC adsorption with either method occurred to all parts of cell surfaces, and there was no special loci for the adsorption. In addition, RBC adsorption was found to occur either being mediated or unmediated by microvilli, regardless of the coating method. The results suggest that surface morphology plays no role in age-related changes in RBC adsorption and the difference between the results with the two coating methods.

Vol.
16
1981

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RELATIONSHIP BETWEEN PHYSICAL ACTIVITY AND LIFE SPAN IN THE ADULT HOUSEFLY, *MUSCA DOMESTICA*

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Vol.
16
1981

INTRODUCTION

IN MAMMALS an inverse correlation seems to exist between basal metabolic rate and maximum life span (Sacher, 1959). The total amount of energy expended per life span in five different species of domesticated mammals has been calculated to be rather constant (200 kcal/g of body weight) (Rubner, 1908). Experimental analysis of the relationship between metabolic rate and life span has been mostly based on effects of temperature on the life span of poikilotherms. These studies led to the concept often referred to as the "rate of living theory", which postulated that life span is dependent upon the rate at which a fixed metabolic potential is expended (Pearl, 1928) with the implication that increase in the metabolic rate shortens life span by accelerating the rate of aging (for review see Sohal, 1976, 1981). Subsequent studies testing this hypothesis employed a variety of temperature regimes, such as transfers from high to low temperature or vice versa at different ages, or daily alternate exposures to high and low temperatures (see Sohal, 1976 for references). However, these studies have, in general, failed to fully support or negate the predictions of the rate of living theory (Maynard-Smith, 1963; Hollingsworth, 1968; Lints, 1971). Possible source of this discrepancy has been ascribed to the widely held, albeit erroneous, assumption that ambient temperature could be strictly equated to metabolic rate (Sohal, 1976; Sohal and Donato, 1979).

The present study was undertaken to reinvestigate the relationship between metabolic rate and life span by employing physical activity rather than temperature as a means to affect the metabolic rate. The specific objectives of this investigation were: (a) to examine the relationship between the level of physical activity of houseflies and life span, and (b) to determine if the natural differences in the physical activity of individual houseflies were related to their life span. The results indicate that life span in the houseflies is strongly influenced by physical activity.

MATERIALS AND METHODS

Following emergence from the pupae, the adult flies were fed on sucrose and maintained at 25°C under 24-h lighted conditions. The level of physical activity of the houseflies was manipulated by excision of wings in 1-day old flies or by varying the size of the housing containers. In order to alter flying activity, in some experiments the flies were put in one-cubic-foot cages where flight was possible, whereas in other cases the flies were singly confined in small bottles, approximately 150 cm^3 in volume, containing a cardboard maze in order to prevent flying. In such bottles the flies could walk but due to inadequate space were unable to fly. For mortality computations, the dead flies were removed every 1–2 days. Student's *t*-test was used to determine the significance of the differences in the life spans of different groups.

Walking and flying activity of the houseflies was measured simultaneously by radar–Doppler instrumentation developed in this laboratory. Applicability of this technique in measuring the physical movement of houseflies and other insects has been reported previously (Buchan and Sattelle, 1979). Houseflies were put in 3-liter

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plastic jars, containing adequate amounts of sucrose and water. The jars were then placed in a marked position, one at a time, within a metal chamber. The movement of the flies within the chamber was detected by a radar module, containing both the receiver and the transmitter, inserted in one of the walls of the chamber. The radar–Doppler device had the capability of detecting the movement of a single fly and distinguishing between the flying and the walking movement (for details of the principle and operation see Buchan and Satelle, 1979).

RESULTS

Only the male flies were used in these experiments in order to avoid the differences which may exist between the sexes.

The relationship between flight activity and life span was initially examined by comparing the life spans of a group of 50 normal flies (group A) to a similar group of experimentally dewinged flies (group B) both of which were confined in one-cubic-foot cages. The median and the maximum life spans of the dewinged flies were about 25 per cent longer (Fig. 1) as compared to the normal winged flies (Fig. 1; $p < 0.05$) which suggested that flight activity has a deleterious effect on the life span.

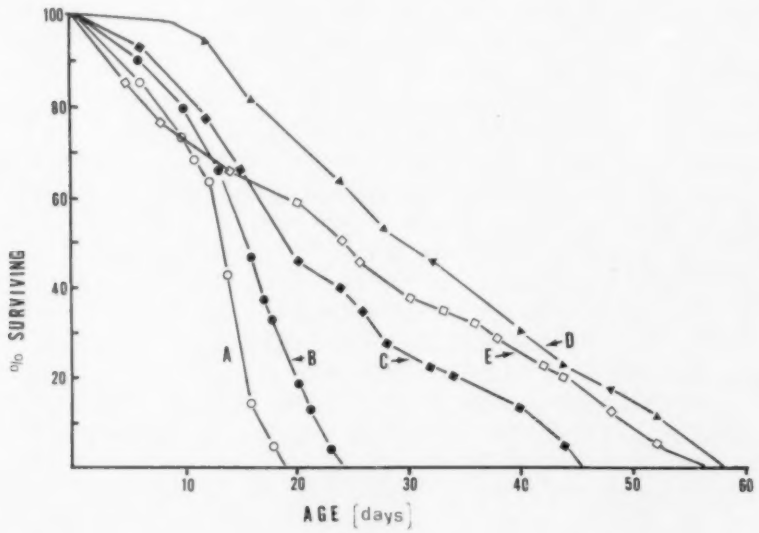

FIG. 1. Survival curves of male houseflies maintained under different experimental regimes. A, 50 normal winged flies in one cubic foot cage; B, 50 dewinged flies in one cubic foot cage; C, one fly per one cubic foot cage; D, one dewinged fly confined in each small bottle; E, one normal winged fly per bottle.

In both group A and group B the male flies were observed to make homosexual copulatory attempts on each other. In order to determine the frequency of homosexuality, copulatory attempts by five different males on other males, in a total population of 100, were recorded during a 1-h period. The average number of attempts was found to be 20 ± 3 and 6 ± 1 in the normal and dewinged flies respectively. The greater frequency of homosexual attempts in the normal winged flies, as compared to the dewinged flies, was probably due to their greater mobility. Dewinged flies were also seen to frequently fall to the floor of the cage, suggesting the inability of the legs to maintain balance in the

absence of the wings. Older dewinged flies also exhibited unsteady walking, probably due to leg damage from the falls.

In order to study the relationship between life span and flight activity, without the complications of homosexuality and leg damage, flies were housed singly, either in one-foot-cubic cages or in small bottles fitted with a cardboard maze where the flies could walk but were unable to fly due to space limitations. The median and the maximum life span of the single winged flies kept in the cages (group C) was significantly longer ($p < 0.01$) than the winged flies kept as a group of 50 (group A) in a similar sized cage (Fig. 1). This would suggest that solitary confinement of flies, where they are free from interaction with other flies, prolongs life span. The median and the maximum life spans of the single winged flies in the bottles (group D) was, however, significantly longer ($p < 0.05$) than the single winged flies in the cages (group C). Shorter life spans in the bottles than in the cages strongly suggests that the absence of flight activity, hence lower rate of metabolic expenditure, prolongs life span. The median life span of the dewinged flies singly confined within bottles (group E) was shorter than winged flies in the bottles (group D) but the maximum life span of the two groups was rather similar (Fig. 1) suggesting the lack of any significant difference in their aging rates. The shorter median life span in group E flies as compared to group D flies can thus be assigned to non-senescent causes.

Vol. 16 1981

The actual levels of physical activity were measured by a radar–Doppler device. In order to determine whether physical activity of solitary flies differed from those kept in a group, walking and flying activity of 10 male flies, 11-days old, each kept singly within a 3-liter plastic jar, were measured separately in each fly. The same flies were later grouped together in one jar of the same size and their physical activity was measured again. Both the walking and the flying activity of the grouped flies increased more than four fold as compared to the aggregate activity of the same flies when kept in solitary condition (Table 1). It therefore seems that interaction among flies within a group greatly increases the level of physical activity.

TABLE 1. COMPARISON OF PHYSICAL ACTIVITY BETWEEN SOLITARY AND GROUPED MALE FLIES

Group	Walking (counts/h)	Flying (counts/h)	*N*
10 single males	1434 ± 70	1646 ± 88	30
Same 10 flies in one group	5911 ± 220	7325 ± 360	25

Additional studies were conducted for determining whether the variations in the life spans of flies belonging to the same group and kept under indentical conditions could be related to the natural differences in the levels of their physical activity. For this purpose 11 male flies were confined singly within 3-liter plastic jars. Walking and flying activity of each fly was measured between 4 and 7 days of age using the radar–Doppler device. This age was chosen on the basis of initial studies indicating that the flies are most active physically at this age. Results on the relationship between the physical activity and the life span of these flies are presented in Fig. 2. Both walking and flying activities appeared to be inversely related to the life span. Although flies with similar longevity exhibited differences in the level of their physical activity, the more active flies had a tendency to die earlier. Statistical analysis of the data showed that walking and flying activities were

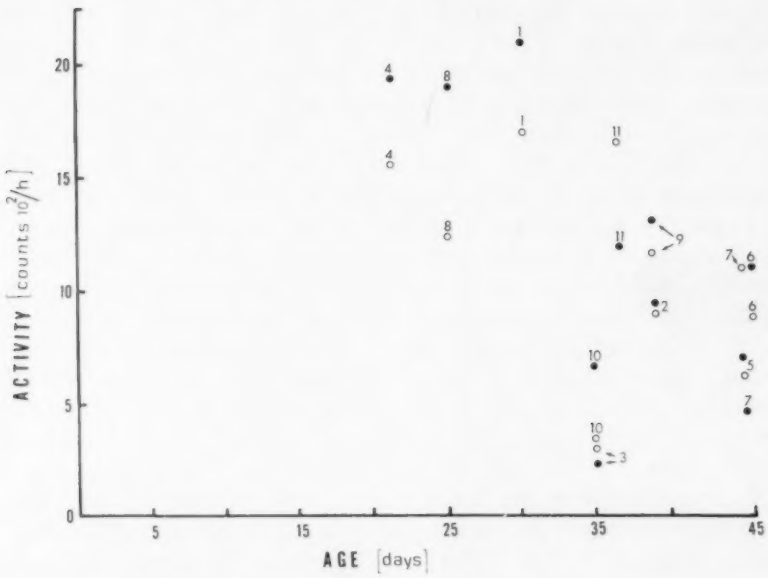

FIG. 2. Relationship between flying activity (solid circles), walking activity (hollow circles) and life span of individual houseflies, each confined in a 3-liter jar.

strongly correlated to each other ($r = 0.812$) suggesting that the flies which flew more frequently also walked more often and vice versa. Correlation between life span and walking was −0.338 and between life span and flying was −0.418. For statistical determination of the relationship between total physical activity, i.e. walking + flying, and life span, canonical correlations were calculated. This statistical technique seeks a linear combination of the variates in the data (Morrison, 1976). The canonical variate (u) was computated as follows: $u = 0.0895 \times$ walking activity $- 1.00726 \times$ flying activity. The canonical correlation was found to be 0.418 which was, interestingly, identical to the correlation between flying and life span. Statistical significance of the correlation between flying and life span was determined by Spearman's rank-correlation coefficient (R), a non-parametric technique (Gibbens, 1971) with the following results: $R = -0.415$; $Z = -1.312$; $p = 0.095$.

DISCUSSION

The results of the present study indicate that life span of the housefly is strongly influenced by the experimental conditions which affect the levels of physical activity. In general, conditions promoting higher degree of physical activity tend to reduce life span. Furthermore, variations in the life span of individual flies, kept under identical conditions, seemed to be related to the differences in the levels of their physical activity.

Several environmental regimes have been reported in the past to affect life span of insects. These include temperature, light intensity, population density and mating activity among others (for review see Rockstein and Miquel, 1973). A common consequence of the various environments which modify life span is the alteration in the relative level of physical activity. For example, the life span of the housefly at 18°C is almost twice that at 28°C (Ragland and Sohal, 1975). During this temperature range flying and walking

activity of the houseflies increased 10- and 15-fold, respectively (Buchan and Sohal, 1981). Similarly, extension of the dark period and the consequent reduction of lighted period during the daily cycle also causes a lengthening of the life span of the housefly (Ragland, 1977) and some other phototropic insects (Rockstein and Miquel, 1973). This is again correlated with the observation that houseflies exhibit little movement in the dark (Buchan and Sattelle, 1979).

Population density has been shown here to have strong effect on the life span of the houseflies. For example, the life span of the winged male flies maintained as a group of 50 in one-cubic-foot cages was shorter than single flies kept in similar-sized cages. The present results have also indicated that grouping of flies greatly increases the levels of walking and flying activity which again suggests an inverse relationship between life span and physical activity. Other factors besides physical activity also seem to play a role in determining the mortality rates of the houseflies. For example, in flies housed in cages, the average maximum life spans of the dewinged flies (group B) were longer than the normal (group A) flies indicating that elimination of flight activity has a beneficial effect on life span. In contrast, in the bottle-confined flies, the average life span of dewinged flies (group E) was shorter than the normal flies (group D) whereas the maximum life span was similar in both groups. An explanation of these results may be that dealation affects the rate of aging by abolishing flight, but also contributes to mortality due to non-senescent causes. It has been pointed out previously by Sacher (1977) that experimental treatments which prolong only the average but not the maximum life span do so without directly affecting the aging rate. Instead, the increased average longevity in such cases is usually due to decreased hazardness of the environment. Indeed, vulnerability of the organisms to disease or accidental injury is determined by the optimacy of their environmental conditions. It is therefore imperative to distinguish between deaths due to senescence and non-senescence related causes. In the context of the present study, removal of wings apparently contributes to non-senescent mortality. Leg muscles of flying insects seem to be ill-suited to sustained activity as they are more dependent on anaerobic than aerobic metabolism (Chefurka, 1965; Sacktor, 1964). As mentioned above, older flies often displayed unsteadiness in walking which was suggestive of leg injuries probably resulting from the falls in the housing containers. Such flies would be more susceptible to death by accidental drowning or starvation.

Whereas the differences in the maximum life spans of different mammalian species have been previously shown to be correlated to the underlying differences in the metabolic rate (Rubner, 1908; Sacher, 1959), the present study demonstrates a similar relationship within the same species and population. Correlation between physical activity and life span suggests that the aging process may be influenced by metabolic rate. The precise mechanism by which this relationship is expressed is presently obscure. However, detrimental effects of oxygen consumption in aerobes such as the generation of highly reactive molecules like singlet oxygen, superoxide, peroxide and hydroxyl radical have been implicated in cellular damage during aging (Fridovich, 1978; Halliwell, 1978). An obvious implication of this hypothesis would be that differences in the life spans among individuals of the same population would depend on the efficiency of their cellular protective mechanisms counteracting oxygen-generated free radicals.

In conclusion, the results of the present study strongly suggest that aging in the housefly is modulated by the rate of metabolic expenditure.

Vol. 16 1981

SUMMARY

The objective of this investigation was to explore the relationship between physical activity and life span in adult houseflies and to determine if the normal variations in the life span observed within a population correspond to the levels of physical activity of the individual flies. The levels of physical activity of male flies were experimentally manipulated by surgical removal of wings and by varying the size of the housing containers. Walking and flying activities of the houseflies were measured, simultaneously, by a radar–Doppler device. Life spans were significantly prolonged following dealation and in response to conditions which decreased the level of physical activity. The median and the maximum life span of flies singly confined in small bottles, where they could walk but were unable to fly, was about 2.5 times longer than those housed in cages where flying was possible. The life spans were longer and levels of physical activity were lower in flies kept under conditions of solitary confinement as compared to those living in a group. Levels of physical activity of individual flies did, in general, correspond to the life span of the flies. Results of this study support the view that life span in the housefly is strongly influenced by the rate of metabolic expenditure.

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PHOSPHOLIPID METABOLISM IN LIVERS OF YOUNG AND OLD FISHER 344 AND SPRAGUE–DAWLEY RATS*

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Vol.
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INTRODUCTION

STORY *et al.* (1976) reported an increase in liver cholesterol and triglycerides with age in Fisher 344 rats. Evidence of phospholipid changes with age (Smith, 1965; Carlson *et al.*, 1968; Eisenberg *et al.*, 1969) has also been reported, in particular, that sphingomyelin increases in human (Buck and Rossiter, 1951; Bottcher and Van Gent, 1961; Rouser and Solomon, 1969; Smith, 1960; Stein and Stein, 1972) and bovine aorta (McMartin *et al.*, 1970). These investigators were careful to distinguish lipid changes due to atherosclerosis from those due to aging. Roelfzema *et al.* (1976) showed that an increase in sphingomyelin occured with age in bovine lens, and Broekhuyse and Kuhlman (1972) demonstrated a similar pattern in human sclera. The sphingomyelin concentration of aorta from different species was shown to be inversely related to the activity of sphingomyelinase in that aorta (Rachmilewitz *et al.*, 1967). Sphingomyelinase, an enzyme that can hydrolyze sphingomyelin to ceramide plus phosphoryl choline, was found to be more active in calf lens than in cow lens (Roelfzema *et al.*, 1973) and sphingomyelin levels were greater in the plasma of old rats than in young rats (Malhotra and Kritchevsky, 1978). We decided it would be of interest to measure hepatic phospholipid levels in aging rats in order to determine whether the changes described above also occur in the liver. At the same time, we assayed several of the enzymes that are important in phospholipid metabolism.

MATERIALS AND METHODS

ATP, serine, coenzyme A, and pyridoxal phosphate were purchased from P–L Biochemicals, Inc., Milwaukee, Wisconsin. Choline chloride and HEPES buffer were obtained from Calbiochem–Behring Corp., La Jolla, California. Dithiothreitol and Tris were obtained from Sigma Chemical Co., St. Louis, Missouri. Tris base was recrystallized from methanol. Sphingomyelin was purchased from Avanti Biochemicals, Inc., Birmingham, Alabama. Boron trifluoride–methanol and phopholipid standards were from Supelco, Inc., Bellefont, Pennsylvania. Fatty acid methyl ester standards were obtained from Supelco, Inc., Applied Sciences, or NuChek Prep., Inc., Elysian, Minnesota. Aquaflex, Flexifluor, and Triton X-100 were scintillation grade reagents from Yorktown Research, Inc., S. Hackensack, New Jersey. (N-methyl-^{14}C)-Sphingomyelin and [9,10(n)-^{3}H]-palmitic acid were purchased from Amersham–Searle, Des Plaines, Illinois, and adenosine-5′-(γ-^{32}P)-triphosphate was purchased from the New England Nuclear Corporation, Boston, Massachusetts. All other chemicals were reagent grade.

All Fisher 344 and Sprague–Dawley rats were obtained from Charles River Breeding Laboratories through the National Institute of Aging, except the three-month-old Sprague–Dawley rats which were obtained directly from Charles River. The rats were maintained on a Purina Chow diet. They were kept for 2 weeks on a 12-h light–dark cycle, with the dark cycle being from 7 a.m. to 7 p.m. Rats were killed by decapitation and their

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livers were perfused with 0.9% saline, frozen in liquid nitrogen, and stored at −20°C. Subcellular fractions were prepared according to the method of Schneider and Vance (1978).

Approximately 2 g of lipids were extracted from rat liver according to the procedure of Folch *et al.* (1957). The chloroform layer was dried on a rotary evaporator, and the lipid extract was redissolved in 2 ml chloroform. An aliquot of the lipid extract was spotted on Silica Gel H plates (Merck), and phospholipids were separated with a solvent system of chloroform/methanol/acetic acid/water, 25 : 15 : 4 : 2.

Fatty acids of phospholipids were methylated with Boron trifluoride–methanol according to the procedure of Morrison and Smith (1964). Samples were dissolved in isooctane and analyzed on a 6 ft column packed with 10% SP-2300 on 80/100 Supelcoport (Supelco) in a Hewlett Packard 5838A Gas Chromatograph. The initial temperature was 195°C. The sample was chromatographed for 28 min.

Sphingomyelinase was assayed in 0.2 ml of 0.1 M potassium acetate (pH 5), with 0.2 mg (N-methyl-^{14}C)-sphingomyelin (1.1×10^6 dpm), and 0.5 mg Triton X-100 (Gatt *et al.*, 1973). Microsomal protein (0.75–1.75 mg) was homogenized in 1% Triton X-100 and added to initiate the reaction. The reaction proceeded for 30 min at 37°C at which time it was quenched with trichloroacetic acid, and sphingomyelin was extracted (Kanfer and Brady, 1969). The trichloroacetic acid layer (2 ml) was diluted with Aquaflex (18 ml) and radioactivity was assayed in a Beckman LS 100 C scintillation counter.

Dihydrosphingosine biosynthesis was measured by a modification of the assay of Braun and Snell (1967). The assay mixture contained the following: 0.1 M HEPES (pH 7.4), 0.05 M NaCl, 0.5 mM dithiothreitol, 1 mg/ml Triton X-100, 1 mM ATP, 6 mM serine, 0.08 mM coenzyme A, 0.5 mM pyridoxal phosphate, 5 mM $MgCl_2$, and 0.5 mM [9,10(n)-^{3}H]-palmitic acid (4.4×10^6 dpm). The total volume was 1.01 ml. Microsomal protein (3–7 mg) in Triton X-100 initiated the reaction which was allowed to continue for 60 min at 30°C. The reaction was quenched with 1.0 ml of 1 M KOH in methanol and heated at 50°C for 10 min. Following centrifugation, the supernatant was placed on a column (0.5 × 2.0 cm) of Amberlite CG400, 200–400 mesh, OH^- form (Mallinckrodt), and the column was washed with 3.0 ml, 0.5 M KOH. The eluant was extracted with 5.0 ml ether (three times), and the combined ether fractions were dried overnight in scintillation vials under a ventilated hood. Flexifluor (10 ml) was added to the vials, and the samples were counted for radioactivity.

Choline kinase was assayed by a modification of the procedure of Infante and Kinsella (1976). In the assay, there were 80 mM Tris (pH 8.0), 30 M choline, 3.75 mM $MgCl_2$, and 2.6 mM (γ-^{32}P)-ATP (1×10^5 dpm) in a total volume of 0.1 ml. The reaction was initiated with 0.2–0.45 mg cytosolic protein. The reaction was allowed to continue for 10 min at 37°C in a capped polypropylene centrifuge tube. The reaction was stopped by placing the tubes in a boiling water bath for 3 min. The tubes were centrifuged and 0.05 ml was placed on an Amberlite CG400 column (0.5 × 2.0 cm). The gel was in the formate form and was equilibrated with 0.25 M pyridine formate (pH 3). The column was washed with 5.0 ml pyridine formate buffer, and radioactivity was counted in the eluant as Cerenkov radiation (Claussen, 1968).

Data were analyzed statistically using a two-way analysis of variance with unequal sample size (Sokal and Rohlf, 1969).

RESULTS AND DISCUSSION

The phospholipid content of rat liver from Fisher 344 and Sprague–Dawley rats of different ages is listed in Table 1. No age-dependent changes in the phospholipid classes are evident though there are strain-related differences. Phosphatidyl choline and phosphatidyl ethanolamine are significantly lower in the Fisher 344 rat. The level of phosphatidyl

TABLE 1. LEVELS IN FISHER 344 AND SPRAGUE–DAWLEY RATS

	Fisher 344*			Sprague–Dawley†		
	3 months	12 months	18 months	3 months	12 months	18 months
Lysophosphatidyl choline	58 ± 36‡	91 ± 36	116 ± 59	35 ± 6	57 ± 20	77 ± 31
Sphingomyelin	112 ± 10	109 ± 2	115 ± 7	122 ± 7	121 ± 2	116 ± 5
Phosphatidyl choline	1460 ± 56	1520 ± 60	1460 ± 35	1650 ± 100	1740 ± 41	1750 ± 68
Phosphatidyl serine–phosphatidyl inositol	338 ± 12	310 ± 41	320 ± 12	366 ± 50	393 ± 19	361 ± 27
Phosphatidyl ethanolamine	737 ± 59	658 ± 47	655 ± 9	868 ± 60	897 ± 46	805 ± 52

*n = 5 (3 months), n = 4 (12 and 18 months).
†n = 4 (3, 12 and 18 months).
‡mg phospholipid/100 g liver ± S.E.M.

serine plus phosphatidyl inositol is also lower in the Fisher 344 rats, although the differences are not as significant statistically ($p < 0.05$) as those of phosphatidyl choline ($p < 0.001$) and phosphatidyl ethanolamine ($p < 0.001$). Uchida *et al.* (1978) have reported that there are no age-related changes in the total hepatic phospholipid content of Sprague–Dawley rats and no difference between Sprague–Dawley and Wistar rats in the total hepatic phospholipid content.

There are no changes in the sphingomyelin content of rat liver either with age or strain (Table 1). Likewise, there are no strain or age differences in the hepatic enzymes of sphingomyelin biosynthesis (dihydrosphingosine) and in the lysosomal enzyme of sphingomyelin hydrolysis (sphingomyelinase) (Table 2).

Plasma sphingomyelin levels of Wistar rats increase with age (Malhotra and Kritchevsky, 1978) and it is possibly that the activities of the hepatic enzymes of sphingomyelin metabolism are different in this strain.

Surprisingly, choline kinase, an enzyme which may be rate controlling in phospatidyl choline biosynthesis (Infante and Kinsella, 1978), is higher in Fisher 344 rats (Table 3). However, the concentration of phosphatidyl choline is higher in the Sprague–Dawley rat. It is not clear at present if the rate determining enzyme of phosphatidyl choline synthesis is choline kinase or CTP-choline phosphate cytidylyl transferase (Schneider and Vance, 1978). Potentially both of these enzymes could control the flux in the pathway of phosphatidyl choline biosynthesis (Infante, 1977). Moreover, it is possible that the activity of cytidylyl transferase could account for the observed differences between the two rat strains in the concentration of hepatic phosphatidyl choline. Infante and Kinsella (1979) have recently reported data that suggest a role for both ethanolamine kinase and CTP-ethanolamine cytidylyl transferase in controlling the biosynthesis of phosphatidyl ethanolamine.

Vol. 16 1981

In explaining the concentration differences in hepatic phosphatidyl choline and phosphatidyl ethanolamine in the Fisher 344 and Sprague–Dawley rats, it may be pertinent to consider the enzymes which hydrolyze these phopholipids, namely phospholipases.

Levels of hydrolytic enzymes may also affect the *in vivo* levels of their substrates. In the case of cholesterol, for example, synthesis is significantly reduced in aging rats (Story *et al.*, 1976; Bloch *et al.*, 1946; Yamamoto and Yamamura, 1971), yet cholesterol accumulates in plasma and tissue (Carlson *et al.*, 1968). Cholesterol 7-α-hydroxylase, the rate limiting enzyme in bile acid synthesis from cholesterol, also decreases (Story and Kritchevsky, 1974). Thus, in assessing accumulation of various lipids it is important to consider not only the biosynthetic pathways, but also the rates of degradation.

The fatty acid composition of hepatic phospholipids from Fisher 344 and Sprague–Dawley rats also indicates no gross changes with respect to either age or strain (Tables 4–8). The ratio of polyunsaturated to saturated fatty acids in phospholipids decreased with age in plasma of Wistar rats (Malhotra and Kritchevsky, 1978).

These studies show a few differences in phospholipid content between Fisher 344 and Sprague–Dawley rats. Levels of phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine plus phosphatidyl inositol are significantly higher in livers of Sprague–Dawley rats, but no age-dependent differences are observed. Despite their lower phosphatidyl choline levels, Fisher 344 rats exhibit consistently higher levels of choline kinase activity. There are no significant differences in levels of hepatic sphingomyelin, activity of sphingomyelinase or in dihydrosphingosine biosynthesis. The ratio of polyunsaturated to saturated fatty acids is higher in the livers of 3-month-old Fisher 344 rats, but the extent of difference declines with age (Table 9).

TABLE 2. HEPATIC ENZYMES OF SPHINGOMYELIN METABOLISM

	Fisher 344			Sprague–Dawley		
	3 months	12 months	18 months	3 months	12 months	18 months
Sphingomyelinase activity*	132 ± 19 (4)‡	118 ± 13 (3)	138 ± 6 (3)	139 ± 24 (3)	152 ± 42 (3)	150 ± 27 (3)
Dihydrosphingosine biosynthesis†	2.49 ± 0.10 (2)	2.09 ± 0.09 (2)	2.25 ± 0.18 (2)	1.91 ± 0.12 (2)	2.26 ± 0.25 (2)	2.28 ± 0.27 (2)

*μg Sphingomyelin hydrolyzed/30 min/mg protein.
†nmoles palmitic acid incorporated/60 min/mg protein.
‡Data are expressed as activity ± S.E.M.; the number of samples in a group is in parentheses.

TABLE 3. HEPATIC CHOLINE KINASE ACTIVITY*

	3 months	12 months	18 months
Fisher 344	30.3 ± 3.8 (3)†	29.5 ± 3.6 (3)	31.9 ± 2.3 (3)
Sprague–Dawley	24.9 ± 2.7 (3)	21.0 ± 3.7 (3)	26.4 ± 2.0 (3)

*nmoles choline phosphorylated/10 min/mg protein.

†Data are expressed as activity ± S.E.M.; the number of samples in a group is in parentheses.

TABLE 4. % FATTY ACIDS OF HEPATIC LYSOPHOSPHATIDYL CHOLINE (± S.E.M.)

	Fisher 344*			Sprague–Dawley		
Acid	3 months	12 months	18 months	3 months	12 months	18 months
16 : 0	26.2 ± 2.8	24.5 ± 1.4	31.6 ± 2.8	23.7 ± 2.3	30.3 ± 3.3	26.0 ± 2.3
18 : 0	53.6 ± 7.8	58.6 ± 11.6	55.4 ± 7.2	66.2 ± 2.6	58.1 ± 3.5	59.5 ± 4.7
18 : 1	6.6 ± 3.6	3.0 ± 1.5	20.1 ± 1.2	7.5 ± 3.2	5.1 ± 3.5	2.3 ± 0.8
18 : 2	2.9 ± 2.3	4.0 ± 2.7	1.7 ± 1.3	0.4 ± 0.3	3.2 ± 1.9	5.4 ± 3.9
20 : 4	10.6 ± 7.9	8.7 ± 8.7	9.4 ± 3.6	2.2 ± 2.2	3.4 ± 3.4	6.9 ± 4.4
22 : 6	tr	1.3 ± 1.3	tr	tr	tr	tr

*n = 5 (Fisher 344, 3 months); n = 4 for all other groups.

TABLE 5. % FATTY ACIDS OF SPHINGOMYELIN ± S.E.M.*

	Fisher 344			Sprague–Dawley		
Acid	3 months	12 months	18 months	3 months	12 months	18 months
16 : 0	11.1 ± 2.9	17.0 ± 2.2	16.5 ± 3.1	14.4 ± 1.9	13.2 ± 3.8	14.0 ± 4.4
18 : 0	6.6 ± 1.3	7.1 ± 1.2	6.7 ± 1.0	7.4 ± 0.8	5.6 ± 1.9	5.6 ± 1.3
18 : 1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.5	0.2 ± 0.5	tr	0.1 ± 0.5
20 : 0	6.1 ± 3.9	2.8 ± 0.9	2.5 ± 0.7	3.2 ± 0.8	2.4 ± 0.5	1.8 ± 0.5
22 : 0	18.3 ± 5.4	13.9 ± 0.3	14.9 ± 0.8	13.8 ± 0.8	13.4 ± 0.6	12.6 ± 0.5
23 : 0	13.0 ± 1.7	13.2 ± 0.9	14.1 ± 1.0	14.4 ± 0.7	14.5 ± 1.4	15.9 ± 2.2
23 : 1	2.2 ± 0.7	2.5 ± 0.1	2.2 ± 0.2	7.1 ± 0.6	5.6 ± 0.2	3.3 ± 1.1
24 : 0	37.4 ± 6.7	39.6 ± 2.0	41.6 ± 2.6	36.5 ± 1.9	42.2 ± 4.5	42.5 ± 6.1
24 : 1	5.1 ± 2.0	3.8 ± 1.5	1.4 ± 0.8	3.1 ± 1.8	3.0 ± 1.8	4.1 ± 1.8

*n = 5 (Fisher 344, 3 months); n = 4 for all other groups.

TABLE 6. % FATTY ACIDS OF PHOSPHATIDYL CHOLINE ± S.E.M.*

	Fisher 344			Sprague–Dawley		
Acid	3 months	12 months	18 months	3 months	12 months	18 months
16 : 0	24.2 ± 2.6	27.1 ± 2.8	26.6 ± 2.5	25.6 ± 2.5	26.2 ± 2.1	25.0 ± 1.6
18 : 0	39.0 ± 3.0	37.5 ± 4.0	37.3 ± 4.4	43.3 ± 4.4	39.7 ± 3.0	37.9 ± 3.5
18 : 1	6.1 ± 0.4	6.6 ± 0.6	6.4 ± 0.6	6.0 ± 0.6	7.0 ± 0.1	7.2 ± 0.6
18 : 2	7.6 ± 0.7	7.6 ± 1.2	6.9 ± 1.6	7.2 ± 1.2	7.8 ± 0.7	6.6 ± 1.0
20 : 4	20.5 ± 3.8	18.3 ± 4.2	19.7 ± 5.4	16.5 ± 4.6	17.6 ± 3.6	20.4 ± 3.7
22 : 6	2.6 ± 0.7	3.1 ± 1.1	3.1 ± 1.2	1.5 ± 0.7	2.0 ± 0.7	2.9 ± 0.5

*n = 5 (Fisher 344, 3 months); n = 4 for all other groups.

Vol. 16 1981

TABLE 7. % FATTY ACIDS OF PHOSPHATIDYL SERINE–PHOSPHATIDYL-INOSITOL ± S.E.M.*

	Fisher 344			Sprague–Dawley		
Acid	3 months	12 months	18 months	3 months	12 months	18 months
16 : 0	4.1 ± 0.4	6.8 ± 1.1	6.9 ± 0.5	4.3 ± 0.4	6.0 ± 0.9	5.6 ± 0.3
18 : 0	69.8 ± 3.2	69.9 ± 6.0	79.1 ± 5.3	77.9 ± 7.6	76.4 ± 3.2	70.5 ± 5.2
18 : 1	1.6 ± 0.2	1.5 ± 0.5	1.6 ± 0.5	1.6 ± 0.3	2.1 ± 0.2	2.3 ± 0.4
18 : 2	0.9 ± 0.1	1.3 ± 0.4	1.2 ± 0.3	2.3 ± 1.0	1.2 ± 0.3	1.7 ± 0.4
20 : 4	23.4 ± 2.8	20.1 ± 5.8	11.3 ± 4.8	13.8 ± 7.6	14.3 ± 3.8	19.6 ± 4.0
22 : 6	0.2 ± 0.2	0.5 ± 0.5	tr	0.2 ± 0.2	tr	0.5 ± 0.5

*n = 5 (Fisher 344, 3 months); n = 4 for all other groups.

TABLE 8. % FATTY ACIDS OF PHOSPHATIDYL ETHANOLAMINE ± S.E.M.*

	Fisher 344			Sprague–Dawley		
Acid	3 months	12 months	18 months	3 months	12 months	18 months
16 : 0	25.8 ± 3.0	30.8 ± 3.8	30.2 ± 1.8	25.2 ± 2.0	26.2 ± 2.3	25.4 ± 1.6
18 : 0	42.1 ± 2.8	40.2 ± 5.2	43.0 ± 4.2	52.3 ± 4.5	43.6 ± 2.9	43.7 ± 5.1
18 : 1	5.6 ± 0.3	8.6 ± 1.4	6.4 ± 1.0	5.9 ± 0.6	6.9 ± 0.4	7.2 ± 0.7
18 : 2	5.6 ± 0.9	6.5 ± 1.8	4.2 ± 0.5	5.0 ± 1.3	6.1 ± 0.7	5.3 ± 1.9
20 : 4	16.6 ± 3.8	10.2 ± 4.0	12.1 ± 3.2	10.5 ± 3.6	13.7 ± 3.6	14.2 ± 3.4
22 : 6	4.3 ± 1.3	3.8 ± 2.9	4.2 ± 2.0	1.5 ± 0.9	3.5 ± 1.4	4.3 ± 1.6

*n = 5 (Fisher 344, 3 months); n = 4 for all other groups.

TABLE 9. RATIO OF POLYUNSATURATED TO SATURATED FATTY ACIDS IN PHOSPHOLIPIDS OF FISHER 344 AND SPRAGUE–DAWLEY RATS

	Age (months)		
	3	12	18
Lysophosphatidyl choline			
Fisher 344	0.72 ± 0.54 (2)*	0.65 ± 0.56 (2)	0.18 ± 0.06 (3)
Sprague–Dawley	0.06 ± 0.04 (2)	0.16 ± 0.08 (2)	0.21 ± 0.06 (3)
Phosphatidyl choline			
Fisher 344	0.52 ± 0.23 (5)	0.49 ± 0.13 (4)	0.54 ± 0.18 (4)
Sprague–Dawley	0.31 ± 0.15 (4)	0.44 ± 0.10 (4)	0.50 ± 0.11 (4)
Phosphatidyl serine plus phosphatidyl inositol			
Fisher 344	0.34 ± 0.07 (5)	0.31 ± 0.10 (4)	0.16 ± 0.06 (4)
Sprague–Dawley	0.23 ± 0.12 (4)	0.20 ± 0.06 (4)	0.30 ± 0.07 (4)
Phosphatidyl ethanolamine			
Fisher 344	0.40 ± 0.10 (5)	0.34 ± 0.16 (4)	0.30 ± 0.10 (4)
Sprague–Dawley	0.24 ± 0.09 (4)	0.36 ± 0.10 (4)	0.37 ± 0.11 (4)

*The number of samples in a group is in parentheses.

The differences in sphingomyelin metabolism observed in aortas (Buck and Rossiter, 1951; Smith, 1960; Bottcher and Van Gent, 1961; Rachmilewitz *et al.*, 1967; Rouser and Solomon, 1969; McMartin *et al.*, 1970; Stein and Stein, 1972) and in the lens of the eye (Roelfzema *et al.*, 1973, 1976; Broekhuyse and Kuhlman, 1972) may, then, be due to metabolic elements of the specific tissue.

SUMMARY

The levels and fatty acid spectra of liver phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, lysophosphatidyl choline and sphingomyelin were compared in 3-, 12- and 18-month-old Fisher 344 and Sprague–Dawley rats. Levels of phosphatidyl choline, phosphatidyl serine plus inositol and phosphatidyl ethanolamine were significantly higher in livers of Sprague–Dawley rats. Although there were strain-related differences in phospholipids, there were no age-related differences. The ratio of polyunsaturated/saturated fatty acids was higher in the phospholipids of young Fisher 344 rats. Choline kinase activity was (20–40%) higher in the livers of Fisher 344 rats, but activities of sphingomyelinase and of dihydrosphingosine biosynthesis were not significantly different from those of Sprague–Dawley rats.

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Vol.
16
1981

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19

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CHANGE OF NUCLEAR PROTEIN PHOSPHATASE ACTIVITY IN THE LIVER OF OLD RATS

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Vol. 16 1981

INTRODUCTION

THE SIGNIFICANCE of nuclear non-histone proteins in the regulation of gene activation is widely recognised (Paul and Gilmour, 1968; Spelsberg and Hnilica, 1970). Several fractions of these proteins are very rich in P (Langan, 1967; Teng *et al.*, 1971) and their regulatory functions correlate with their degree of phosphorylation (Shea and Kleinsmith, 1973; Kleinsmith *et al.*, 1976). During aging the phosphorylation and dephosphorylation of nuclear non-histone proteins (NHP's) show a definite pattern (Kanungo and Thakur, 1977; Szeszák and Viticchi, 1977). As a characteristic feature of the increased P turnover, an increased nuclear protein phosphatase (NPP-ase) activity for endogenous substrates with advancing aging in rat liver cell nuclei was detected (Szeszák and Viticchi, 1977). The present paper describes some further characteristic changes of NPP-ase (EC 3.1.3.16) activity occuring in nuclear NHP preparations of young and old rats when measured with different exogenous—nuclear phosphoprotein (NPP) and casein—substrates.

METHODS AND PROCEDURE

Most of the methodical details of these experiments are given in a preceding paper (Szeszák and Pinna, 1980).

Animals, preparation of nuclei

Male Wistar rats were used throughout the experiments. They were kept on normal diet and starved for 24 h before killing by cervical dislocation. "Young" and "old" animals correspond to 4–6 and 24–28 months of age, respectively. Liver cell nuclei were isolated by the method of Blobel and Potter (1966) and washed with Triton X-100 (MacGillivray *et al.*, 1972). All the procedures were carried out at 0–4°C unless indicated otherwise.

Non-histone protein preparation

Nuclei of one liver were suspended in a 2 ml system containing 0.03 M Tris–HCl, pH 7.5; 0.25 M sucrose; 0.025 M NaCl; 0.005 M Mg acetate; 0.01 M Na_2SO_3 and 0.5 mM diisopropyl fluorophosphate (DFP) and incubated at 37°C for 10 min. This step has been found to be efficient in removing cytoplasmic contaminants from nuclear preparations (Viticchi and Szeszák, 1979). After centrifugation the nuclear pellets were washed three times with 7 ml of 0.14 M NaCl–0.02 M Tris–HCl, pH 7.5, by magnetic stirring for 20 min and centrifugation. The chromatin was extracted in 2 ml of 1 M NaCl–0.02 M Tris and the undissolved material was removed by centrifugation. Nucleohistone was precipitated by dilution to 0.4 M for NaCl adding 0.02 M Tris dropwise to the system. Nucleohistone was pelleted at 50,000 rev/min for 70 min. To remove the residual histones, 1.2 g of Bio-Rex 70 were added and stirred for 10 min. After centrifugation the supernatant was drained and filtered through cotton wool. NHP preparations, distributed in several portions, were stored in liquid N_2.

Labelled substrates

NPP substrate was prepared according to the method of Langan (1967) and Gershey and Kleinsmith (1969) as described before (Szeszák and Pinna, 1980). The specific radioactivity of the labelled NPP product was

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3000 dpm/μg protein. Hammersten casein was labelled by rat liver protein kinase TS as described previously (Meggio *et al.*, 1977). The specific radioactivity of casein was 2000 dpm/μg protein.

Phosphatase assay

The system contained in 200 μl: 0.04 M NaCl; 0.005 M Mg acetate; 0.001 M $MnCl_2$ and 0.025 M glucose. The pH of the system was adjusted as follows: the samples always contained 0.025 M Tris–HCl, pH 7.5. To 6.7 the pH was lowered by adding increasing amounts of pH 4–6 range Ampholine Carrier Ampholyte and down to pH 5.5 by adding increasing amounts of 0.01 M HCl to the pH 6.7 system. The concentration of NPP and casein substrates exceeded the *Km* values determined at pH 6.7. Substrate preparations were treated at 65°C for 5 min to inactivate any endogenous protein phosphatase (PP-ase) activity. If not specified otherwise, the enzyme contents and incubation times at 37°C were chosen so that the amount of ^{32}P orthophosphate liberated was kept below 10 per cent of the input. The liberated ^{32}P orthophosphate was extracted by the method of Martin and Doty (1949) with some modification and radioactivity measured in Packard liquid scintillation spectrometer using Triton cocktail.

Protein determination was done by the method of Lowry *et al.* (1951).

Materials

[γ-^{32}P]ATP was from Amersham (U.K.), unlabelled ATP from Fluka (Switzerland), Tris from Sigma (Trizma, U.S.A.), NaCl from BDH (AnalaR, U.K.), casein from Merck (Germany), DFP from Serva (Germany), Ampholine Carrier Ampholyte from LKB (Sweden).

RESULTS

Some characteristic features of nuclear protein phosphatase examined in young and old rats

NPP-ase activity can be measured with NPP substrate at a broad range of pH in NHP fractions of young and old rats (Fig. 1). The curves are of similar pattern, show two, not very much pronounced maxima. The higher values of old animals were reproducible in separate experiments.

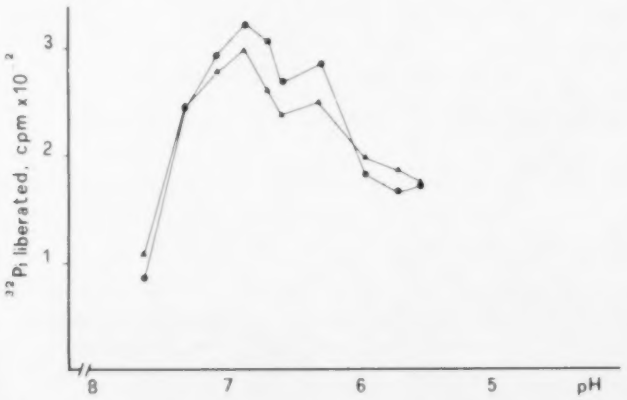

FIG. 1. NPP-ase activity measured at different pH values in NHP fractions prepared from liver cell nuclei of young and old rats. Pairs of NHP fractions from young and old animals were prepared in parallel. Their NPP-ase activity on NPP substrate was measured in the same experiment. ▲, young; ●, old.

Presumably it is conceivable that a higher proteolytic activity in NHP fractions of old animals might result in a more extensive fragmentation of NPP substrate and consequently an increase in its accessibility for NPP-ase. Therefore the time course of NPP-ase action and the effect of DFP on it were also examined (Fig. 2). DFP is a potent inhibitor of chromatin proteases (Carter and Chae, 1976). It exerted a partial inhibitory effect on NPP-ase activity, particularly at pH 6.1, however, it did not show any age-related effect.

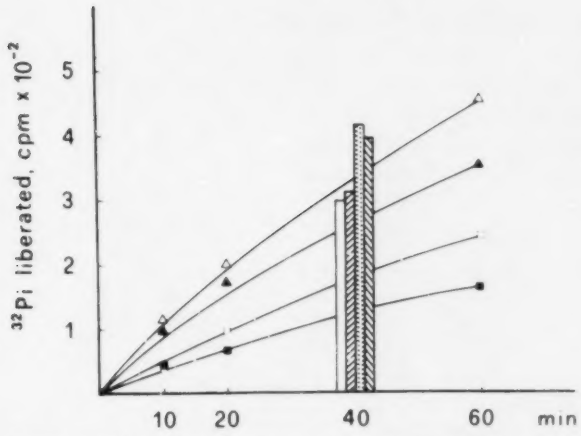

FIG. 2. Enzymatic dephosphorylation of NPP substrate by fractions of NHP from young and old rats; the effect of DFP. Curves: NPP-ase activity measured at pH 6.1; columns: activity measured at pH 6.7 during 40 min of incubation. △, HNP from old animals without DFP; ▲, old with DFP; □, young without DFP; ■, young with DFP; open column: young without DFP; column hatched upward: young with DFP; dotted column: old without DFP; column hatched downward: old with DFP.

Vol. 16 1981

The age-related differences of NPP-ase activity are pronounced at pH 6.1 as well as at pH 6.7. Therefore, it is unlikely that any differential proteolytic effect exerted on the substrate by NHP fractions would be responsible for the age-related differences observed. By exhaustive dephosphorylation of NPP substrate (Fig. 3) it was shown that not all the incorporated ^{32}P is accessible for NPP-ase. In the presence of excess amount of NHP preparations from young animals about 25 per cent of ^{32}P can be liberated. With NHP preparations from old rats this proportion is over 45 per cent.

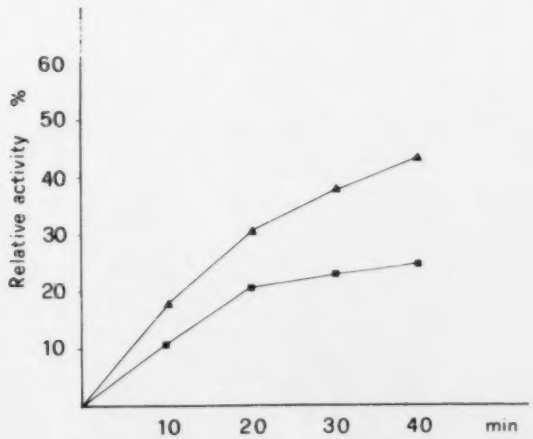

FIG. 3. Exhaustive dephosphorylation of NPP substrate by NHP fractions from young and old rats at pH 6.7. The values are expressed in per cent of input radioactivity. The samples contained 5 μg of NPP substrate and 20 μg of NHP fraction. In four experiments carried out with different pairs of NHP fractions and different batches of NPP substrate the maximum liberation levels of ^{32}P obtained for young and old animals fell between 20–30% and 40–50%, respectively. ■, young; ▲, old.

Substrate specificity of NPP-ase in young and old rats

One interesting property of NPP-ase was revealed when its activity was measured with labelled NPP and casein substrates in parallel in NHP fractions of old and young rats (Table 1). PP-ase activity for casein and NPP substrates changes by an opposite manner in old animals in comparison with young ones and it is only the activity for NPP substrate which is higher in old animals while the casein PP-ase activity decreases. The tendency of the age-related change of NPP-ase activity for NPP substrate, when measured at pH 6.7, is similar to that measured at pH 6.1.

TABLE 1. CHANGE OF NPP-ASE ACTIVITY IN OLD RATS

	Substrate								
	Casein, pH 6.1			NPP, pH 6.1			NPP, pH 6.7		
Source of NHP	cpm/10 μg NHP Protein	S.E.	Young / Old	cpm/10 μg NHP Protein	S.E.	Young / Old	cpm/10 μg NHP Protein	S.E.	Young / Old
Young	2081	± 168	1.358	2031	± 130	0.777	4008	± 115	0.669
Old	1532	± 87		2613	± 197		5985	± 389	

NHP fractions were prepared individually from 6 young and 6 old animals under identical experimental conditions. NPP-ase activity was measured with labelled NPP and casein substrates in the same experiment at pH values as indicated. The values were statistically analysed by the Student *t*-test.

Significance levels: $p < 0.02$; $p < 0.05$; $p < 0.001$.

Lineweaver–Burk's plots taken up with NPP and casein substrates at pH 6.1 (Fig. 4a and b) are rectilinear with fairly good regression coefficients.

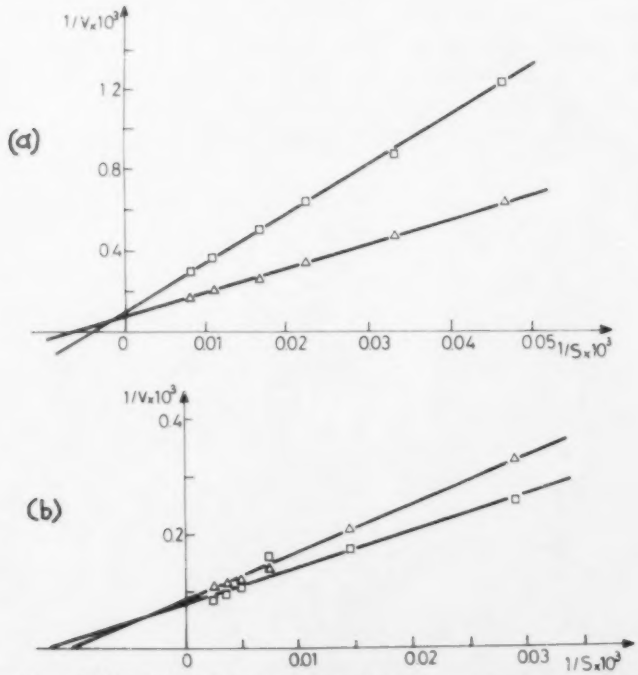

FIG. 4. NPP-ase activity of NHP fractions from young and old rats measured with NPP and casein substrates at pH 6.1. The numerical data of the Lineweaver–Burk plots were taken from the input and liberated ^{32}P cpm values. □, young; △, old. (a) NPP substrate. Regression coefficients for young and old are $r_y = 0.9951$ and $r_o = 0.9973$, respectively. (b) Casein substrate. Regression coefficients for young and old are $r_y = 0.9590$ and $r_o = 0.9929$, respectively.

DISCUSSION

Protein phosphatase activity in cell nuclei and nuclear subfractions was detected with endogenous substrates (Olson and Guetzov, 1976; Szeszák, 1976; Periasamy *et al.*, 1977) and it was also partially purified (Szeszák and Pinna, 1980). State of phosphorylation of phosphoproteins is usually controlled primarily through the effect of protein kinases (Fisher *et al.*, 1971) which have more than 1000-fold higher activity than the corresponding PP-ase. On the other hand, in isolated rat liver nuclei the dephosphorylation rate of proteins overcomes the rate of their phosphorylation (Szeszák, 1976). It may show the particular significance of protein phosphatases involved in the turnover of P bound to nuclear proteins. This finding prompted us to study NPP-ase in relation with the aging.

Our data reveal the complexity of NPP-ase activity in rat liver cell nuclei. It is apparent from the broad biphasic curve obtained for NPP-ase activity at different pHs (Fig. 1) and from the fact that it changes by an opposite manner with age when measured for NPP and casein substrates. Several enzymological data are also in favour of the non-identity of PP-ases active for NPP and casein (Szeszák and Pinna, 1980). One has also to take into account the heterogeneity of NPP substrate (Langan, 1967).

Vol. 16 1981

The dephosphorylation is a limited process in our system. About 90 per cent of ^{32}P in our NPP substrate is incorporated into serine–P bonds and these are the sites of the NPP-ase action (Szeszák and Pinna, 1980). Not all of these serine–P bonds are accessible for NPP-ase(s). In the presence of excess enzyme the dephosphorylation curves tend to reach a plateau value (Fig. 3). The main age-related changes observed in our system are: (i) An increase of NPP-ase activity with NPP substrate in old rats when measured either at pH 6.1 or 6.7, (ii) a decrease of casein phosphatase activity measured simultaneously (Table 1); (iii) a higher level of maximum dephosphorylation in old rats (Fig. 3). The question is whether the increase of NPP-ase activity with NPP substrate reflects a quantitative or also a qualitative change in old rats. The higher dephosphorylation maximum would show a qualitative change of NPP-ase in old animals, however, linear curves obtained in Lineweaver–Burk's plots (Fig. 4a and b) show rather a change of quantitative character. The problem shall be clarified by further purification of the components. At present, the phenomenon may be explained also by assuming the presence of some factor, other than NPP-ase, in NHP fractions of old animals which is capable of making further serine–P sites accessible for NPP-ase and it would be present in excess at all the substrate : enzyme ratios examined.

Considering earlier data too, the significance of the phosphorylation of nuclear non-histone proteins in the process of aging could be hypothesized as follows: the NHP's contain regulatory elements of the genetic transcription (Paul and Gilmour, 1968; Spelsberg and Hnilica, 1970) and of these, the more phosphorylated forms are the more active ones (Shea and Kleinsmith, 1973; Kleinsmith *et al.*, 1976). The main characteristics of the turnover of protein bound P in cell nuclei are that in middle and old ages the nuclear protein kinase and phosphatase activity increase while the absolute amount of protein bound P begins to decrease only in old age (24–28 months, Szeszák and Viticchi, 1977). The increased turnover of P may represent some compensatory mechanism against failures of the transcriptional activity in the nuclei of middle-aged rats.

The present data provide further information about the dephosphorylation step of the turnover of protein bound P in cell nuclei and show NPP-ase to form a complex system consisting of enzymes of different substrate specificity. The activity increase of NPP-ase in old age is not of a general character, since it concerns only some kind(s) of NPP-ase being active for NPP substrate but not for casein.

SUMMARY

Nuclear protein phosphatase (NPP-ase) activity was measured in non-histone protein (NHP) preparations obtained from liver nuclei of young and old rats with exogenous substrates labelled with ^{32}P. NPP-ase activity can be measured in a rather broad range of pH between 5.5 and 7.5 and it shows several characteristic differences between young (4–6 months) and old (24–28 months) animals: (a) under the effect of NPP-ase a limited dephosphorylation of nuclear phosphoprotein (NPP) substrate ensues reaching a 25 per cent liberation maximum of incorporated ^{32}P in the case of NHP preparations from young animals; the same liberation maximum is over 45 per cent in the case of old animals, (b) an age-related change of NPP-ase activity is assumed to occur in old rats in comparison with young ones. The NPP-ase activity measurable for its homologous—NPP—substrate increases in old animals while there is a concomitant decrease of casein phosphatase activity. The possible implication of the phosphorylation of chromosomal non-histone proteins in the cellular aging is discussed.

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ON RATIONAL USE OF THE CROSS-SECTIONAL METHOD IN ANALYSIS OF THE DYNAMICS OF BIOLOGICAL INDICES WITH AGING

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Vol. 16 1981

INTRODUCTION

IN THE gerontological investigations the dynamics of various biological indices with aging is frequently studied with aid of the cross-sectional method. Despite the drawbacks related with the inability to consider the individual age shifts (Comfort, 1964), the cross-sectional method is convenient as within the relatively short study duration it allows to follow the dynamics of average biological indices of aging of a certain species or population. In some investigations where in keeping with the experiment requirement it is impossible to keep the animal alive the cross-sectional method is the only approach. Therefore, further elaboration and improvement of this method seems worthwhile.

The cross-sectional method has a peculiarity that makes the interpretation of the results obtained difficult, i.e., the age dynamics is not a mere reflection of the physiological, but also of the populational-statistic changes linked with the differential mortality due to which only the individuals with the most positive values of the vital parameters survive to old age (Exton-Smith, 1970; Voitenko, 1973; Pakin *et al.*, 1978; Sachuk, 1978). The above is especially important at late stages of ontogenesis where age mortality rates reach maximal values.

There are publications in literature showing an attempt to overcome the existing difficulties (McGilchrist and Simpson, 1979; Donato *et al.*, 1979), but the problem is as yet far from being solved.

The purpose of the present paper is to elucidate some peculiarities of the cross-sectional method and to offer one of the ways of its rational application.

The analysis of consideration of the data on differential mortality rates, using the proposed principle, has been made on the example of age dynamics of the human peripheral blood lymphocyte PHA-transformation.

MATERIAL AND METHODS

The model construction is based on analysis of the hypothetical variants of the influence of differential mortality upon average values of the indices, consideration of the possible results of differential mortality with a use of life tables, and statistical estimation of the influence of a parameter on the organism's viability.

In illustrating this model on the example of PHA-transformation the author's data obtained on peripheral blood lymphocyte cultures of human subjects aged 45 (43–47) yr, $n = 24$; 50 (48–52) yr, $n = 21$; 55 (53–57) yr, $n = 19$; 60 (58–62) yr, $n = 32$; 65 (63–67) yr, $n = 38$; 70 (68–72) yr, $n = 36$; 75 (73–77) yr, $n = 28$; 80 (78–82) yr, $n = 16$; 85 (83–87) yr, $n = 10$, were used.

The lymphocytes were cultured for 72 h at 37°C in medium 199 containing 25% of serum and PHA "Reanal" in the dose of 2.0 μl/ml. Lymphocyte concentration was 3.10^5 per 1 ml of culture. Four hours prior to the fixation 0.5 μCi/ml of ^{3}H-thymidine (specific activity 15 Ci/mmole) was added. Acid insoluble precipitate was placed on the millipore filters; radioactivity was measured by means of liquid scintilating counter "Intertechnick SL-30".

STATISTICAL CONSIDERATIONS

It is known that the aging process if characterized by a decline of organism's functional possibilities that can be expressed both in an increase of the absolute values of the indices, and in their decrease. Let us assume that age dynamics would show itself in a decreased value.

For the solution of the possible results of the action of the populational-statistic effect, we discuss the two extreme hypothetical cases: (1) age-related change of the value of the hypothetical variable x does not influence the organism's viability v and thereby the mortality rate, i.e. $\tau_{vx} = 0$ where τ—a correlation coefficient; (2) change of the variable value does influence the organism's viability functionally, i.e. $v = f(x)$.

1. In case of $\tau_{vx} = 0$ the elimination (drop-out from the population) because of death will take place irrespective of the index value, i.e. it will be accidental in relation to the variable under study. Thus mortality will introduce accidental distortions of average values which will tend to a zero with increasing sample size. Hence, at the absence of the influence of age shifts of the variable on the mortality rate, the populational-statistic effect would not operate and the age dynamics measured cross-sectionally would only reflect age physiological shifts.

2. In case of $v = f(x)$ the elimination from the sample due to death would take place in subjects with the lowest values of a given variable. We discuss the possible consequences of this situation. Let us assume that in some sample of subjects aged y the following hypothetical values of the variable x have been obtained: 1000, 900, 800, 700, 600. Their average value $M_{y(x)}$ is 800. Then because of a decrease in the indices occurring with age at age $z > y$ these values would become 970, 870, 770, 670, 570. In a given example in case of elimination from the sample of an individual having the lower value of the variable $x_{\min} = 570$, the value of $M_{z(x)}$ would be $820 > M_{y(x)}$, i.e. average value of the variable in older age group would be higher, as compared with the younger group, that may make a wrong impression about the character of changes in this variable with aging.

Thus if $\tau_{vx} = 0$, the population-statistic effect S is absent, $S = 0$; if $v = f(x)$, then $S = S_{\max}$. From the analysis of the above two extreme hypothetical variants it is possible to postulate: the greater the influence of the variable on the organism's viability, the more marked the populational-statistic effect is, i.e. the greater distortion of the physiological age dynamics would be obtained in the investigations employing a cross-sectional method. Such situation would occur both in the case of a direct influence of the variable on the organism's viability, and in the case of a correlation between the variable under study and other vital parameter (or parameters).

Following from the fact that the influence of the populational-statistic effect on average values of the indices is conditioned by elimination with aging of subjects with less favourable values of the vital variables, it may be concluded that in order to obtain true (physiological) age dynamics of indices one should make a selective recruitment of subjects in younger age groups and compose the sample of only those subjects who possess a potential possibility to survive to the older age group.

In the above hypothetical case $v = f(x)$ there is an unequivocal relationship: the lower the value of the index under test, the lesser viability of the individual, i.e. the higher probability of his death. Hence, in case of deaths occurring within the age interval studied the individuals with the lowest values of a given variable would not survive into the old age group. Therefore, in order to obtain a true (physiological) dynamics of the variable under test one should exclude this particular part of the subjects from the younger age group while making the calculation of the averages of this variable.

The question of how many subjects with the minimal values should be excluded from the younger age group sample can be solved with the help of the data on probability of survival p taken from life tables which are generally calculated for certain human populations in relation to age and sex.

In our considerations the life tables for the population of the Ukrainian SSR with five-year intervals from 1961 to 1980 were used (Korchak-Chepurkovsky, 1970). For calculation simplification the rounded values of probability of survival irrespective of sex and annual-wise dynamics were taken.

From the above tables it was thus found through the standard estimation that the probability of survival, say to 85 years, increases with increasing age (Fig. 1). In a given case to the control age of 85 years survive 29.2% of 40-year old subjects, 29.6% of 45-year olds, 30.3% of 50-year olds, 31.4% of 55-year olds, 32.7% of 60-year olds, 34.2% of 65-year olds, 39.5% of 70-year olds, 47.4% of 75-year olds, 63.2% of 80-year olds and, naturally, 100% of the 85-year old individuals.

Vol. 16 1981

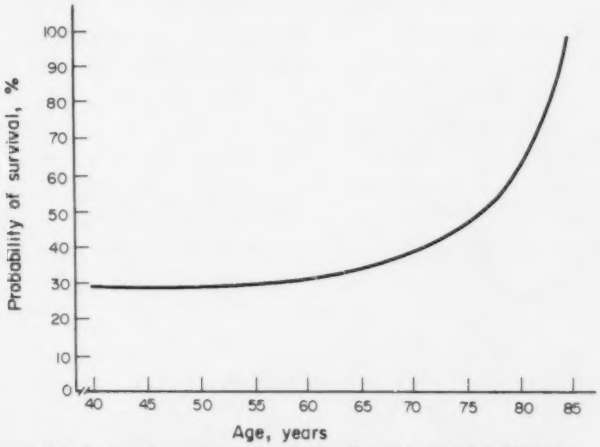

FIG. 1. Age dynamics of the probability of survival to 85 years.

Based on earlier assumed $v = f(x)$ it appears that in each age group the respective number of precisely those subjects survive to the control age who show higher values of a given variable. So, in order to obtain the physiological age dynamics of the variable tested, while making the estimates of the averages one should exclude the remaining number of observations with the lowest values from each sample. That is in case of $v = f(x)$ after ranking the variable values in diminishing succession one should find the average value M of the variable x at age y from the formula:

$$M^{z}_{y(x)} = \frac{\sum_{i=1}^{n_1} x_i}{n_1}; \; n_1 = [N \cdot p],$$

where $x_1 \geqslant x_2 \geqslant \ldots \geqslant x_{n_1}$, p—probability of survival from age y to age z, N—the number of observations in the sample, or after ranking the variable values in increasing succession, from

$$M^{z}_{y(x)} = \frac{\sum_{i=1}^{N} x_i - \sum_{i=1}^{n_2} x_i}{N - n_2}; \; n_2 = [N \cdot q],$$

where $x_1 \leqslant x_2 \leqslant \ldots \leqslant x_{n_2} \leqslant \ldots \leqslant x_N$, q—probability of subjects at age y to die (not to survive) to age z; $q = 1 - p$.

We discussed the hypothetical situation when there is a functional relationship between the variable value and the viability. However, it is known that biological relationships are not functional, but correlative. In our case this is because the viability depends on the set of k variables, i.e. $v = f(x^{(1)}, x^{(2)}, \ldots, x^{(k)})$, and at a fixed value of one variable, the values of other variables may vary and thereby introduce changes in the viability. Since the influence of any variable on the viability cannot be 100% (functional), but has an intermediate value from 0 to 100 per cent, we should take into account in our considerations the share of contribution made by the variable to the viability and age elimination process.

As was mentioned above, in case of the absence of the influence of the variable on the organism's viability, the populational-statistic changes would not alter the age dynamics, i.e. for the estimates of physiological age shifts of the variable it is necessary to take the whole sample. In case of the functional relationship between the variable value and the viability, one should take the part of the sample which corresponds to the probability of survival. Hence, the greater the influence of the variable on the viability, the larger part of the observations should be excluded from the sample. In the first approximation one can assume the relationship between the variable value and the viability to be a linear dependence and thus exclude from the sample the corresponding fraction of the worse values on the basis of the probability of survival and the share of the variable effect on the viability. That is, at positive correlation between the variable under study and the viability the average value should be found in the range in increasing x succession from the formula:

$$M^z_{y(x)} = \frac{\sum_{i=1}^{N} x_i - \sum_{i=1}^{n_3} x_i}{N - n_3}; \quad n_3 = [N \cdot q \cdot \gamma],$$

where $x_1 \leqslant x_2 \leqslant \ldots \leqslant x_{n_3} \leqslant \ldots \leqslant x_N$, γ—share of the variable influence. In case of the negative correlation the average value of the variable should be found from the same formula with x values ranged in diminishing succession.

The averages thus obtained would reflect the dynamics of the variable in aging subjects who survive to age z (in our hypothetical example to 85 yr).

It should be noted that physiological age dynamics of the values of the vital variable cannot reflect a change of this variable in some averaged individual (for example, man) in general, but it can only be referred to a concrete organism or a group of organisms of a given species surviving to a definite age. Otherwise, the presence of subjects with shorter life-span in the younger age groups would make the populational-statistic effect operate.

One of the approaches to assessing the contribution of the variable to the viability can be a use of the variance analysis. The variance analysis allows to single out the share of the influence of a concrete variable among other influences (whose total value makes 100%) from the formula (Lakin, 1973):

$$\gamma = 1 - \frac{D_1}{D_2} Q,$$

where γ—share of the variable influence, D_1—variation values of the variable within separate groups, i.e. near group averages (accidental variance), D_2—variation values of

the variable around the arithmetic mean of all observations (total variance), Q—correction coefficient for the displacement of sample value y from the populational parameter.

In the gerontological investigations the variance analysis is often used for studying the effect of age on the variable value. For the solution of our task we may ask this question the other way: what is the share of influence of a concrete variable on the average age in the sample under test.

In the biological sense the question is asked in such a way because we mean a biological, but not a calendar, age and it depends not on the number of years lived, but on the biological index value. As is known, the biological age is a measure of the potential functional possibilities of an organism, i.e. a quantitative criterion of the viability. And, if the assessment of the biological age of a concrete individual is now far from being a solved task, the average biological age of the representative sample is a known value, it equals the average calendar age. That is why the calendar age can be used in estimation of the share of the variable tested in the average value of the biological age of the sample, i.e. its viability.

It should be noted that the share value of the variable in the organism's viability as assessed by means of the variance analysis does not necessarily reflect a direct physiological effect of this variable on the viability, but can be a result of its correlation with other vital parameters, i.e. it represents a statistical value.

Vol. 16 1981

USE ON THE EXAMPLE OF LYMPHOCYTE PHA-TRANSFORMATION

The proposed principle has been used for the assessment of physiological age dynamics of human peripheral blood lymphocyte PHA transformation based on our data obtained cross-sectionally. The influence of PHA-transformation on the viability as found by the variance analysis makes ~ 11 per cent. Figure 2 shows that within a range of 45–70 y the

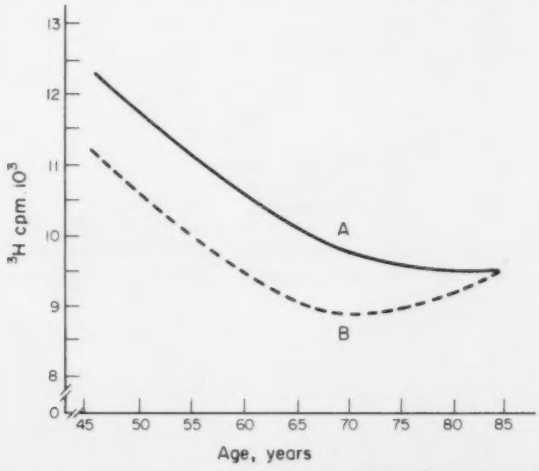

FIG. 2. Age dynamics of the lymphocyte PHA-transformation (^{3}H thymidine incorporation) with regard to the populational-statistic effect (A) and without regard to the populational-statistic effect (B).

dynamics of PHA-transformation, with/without regard to the populational-statistic effect, is qualitatively of the same character, and differs only quantitatively. Within age interval of 70–85 y there are significant differences in that age dynamics of PHA-transformation shows an increase of its value, when determined without regard to the

populational-statistic effect, and a decrease when this effect is taken into account, the decrease being less marked as compared with young age.

CONCLUSION

The described model does not solve all problems concerning the cross-sectional method, but it can be one of the approaches to their solution. It should be noted that determination of the variable contribution to the organism's viability is a quite complicated task. Application of the variance analysis allows to obtain a precise estimation of the variable contribution to the viability in case of a stable value of the variable influence and in case of the linear dependence on age. With non-linear dependence the variance analysis allows to estimate an average value of the variable influence for the whole age range under study. In this case the estimation of precise values of the variable influence at separate stages of ontogenesis presumably requires another approach. Besides, the consideration of individual and constitutional peculiarities of the rate of changes in the indices with aging requires a special study.

SUMMARY

The presence of populational shifts conditioned by the differential age mortality can influence the character of age dynamics of the indices obtained by means of the cross-sectional method. The result of this influence (populational-statistic effect) becomes greater, the higher the correlation between the variable under study and the viability of the organism is. The model is proposed in which the use of the probability of survival and the variable influence on the viability can be a helpful tool for estimation of the populational-statistic effect and obtaining the physiological age dynamics of the indices.

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AGE DEPENDENT ULTRASTRUCTURAL CHANGES IN DIFFERENT ORGANS OF THE MECOPTERAN FLY *PANORPA VULGARIS*

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Vol.
16
1981

INTRODUCTION

DURING the last years a number of observations on age dependent histological changes of insects were published. These observations were mainly restricted to very few species of dipterans (*Drosophila, Musca, Phormia, Calliphora*) and in most cases dealt only with the dystrophy of flight muscle. (Takahashi *et al.*, 1970*a, b*; Miquel, 1971; Webb and Tribe, 1974; Johnson and Rowley, 1972; Rockstein and Bhatnagar, 1965; Sohal, 1976; Simon *et al.*, 1969; Turturro and Shafiq, 1979).

The results of these papers are often contradictory not only between different species but within one species alone, reaching from the observation of "total degeneration" (Simon *et al.*, 1969; Miquel, 1971; Takahashi, 1970*b*) to "poorly developed changes" (Rockstein *et al.*, 1975; Sohal, 1976; Webb and Tribe, 1974) in *Musca* and *Drosophila* respectively, and own unpublished studies on *Phormia terrae novae*. Therefore it seemed desirable to look from a comparative viewpoint for age related histological changes in several organs of a member of another group of insects with other functional designs. The mecopterans have never before been the subject of ultrastructural and biochemical investigations, not to mention ageing studies. They have in contrast to the dipterans a synchronous flight muscle.

The following paper describes fine structural alterations in the flight muscle, fat body, malpighian tubules, salivary glands and brain of the scorpion fly *Panorpa vulgaris* (*Mecoptera, Panorpidae*).

MATERIALS AND PROCEDURE

Adult imagos were maintained in the laboratory on long day (18 : 6 h) conditions at 23 ± 2°C and 60–75% R.H. in cages (40 × 20 × 35 cm) with 50 individuals each and fed *ad lib.* with mealworm larvae. Under this treatment males and females reach a maximum life span of about 110 days which is in accordance with free living populations. Life expectancy at the day of emergence was 61–63 days for both males and females.

Panorpa larvae were reared in plastic bags with moist peat-soil after sampling the egg-clusters from the same substrate and fed with mealworm larvae. Under the same conditions as for the adults the larvae (1 i) hatch after 6 days, pass through the 4 larval stages in 3 weeks and emerge after an additional 3 weeks. The adult males emerge 3 days after the females.

Male specimens of different ages were chosen as indicated below. The organ samples were prepared in buffered glutaraldehyde (cacodylate-buffer 0.1 M, pH 7.25) and fixed 1 h in glutaraldehyde–osmiumtetroxide (Franke *et al.*, 1969). Depending on the object a post-fixation of 2–20 h was necessary. The samples were oriented in agar, after the usual dehydration-procedure embedded via propyleneoxid in Epon (Richardson *et al.*, 1960) and sectioned with an OMU-3 Reichert ultramicrotome. The ultrathin sections were contrasted with uranylacetate and lead citrate (Venable and Coggeshall, 1965). All photographs were taken with a Zeiss EM 9-S2 elmiscope.

A morphometric determination of the portion of the sarcoplasmic reticulum in the flight muscles was carried out by plotting the number of vesicles against the occupied surface area.

RESULTS

All the studied tissues except for the ommatidia show remarkable and often spectacular degenerative histological changes in specimens of old age.

Flight muscle (Fig. 1 A–K; Fig. 2 A–F)

The flight muscle of *Panorpa* shows the typical appearance of the synchronous type with well developed sarcoplasmic reticulum and large mitochondria lying in the space between the myofibrills. The actin and myosin filaments and the other bands of the fibrillar muscle are clearly visible. Muscles of freshly emerged (1-day), 17-day, 54-day, 63-day and 91-day old adults were compared and show striking variations. On sections of "one day" muscle one can see bright areas which underwent dissolution, lying between fully intact and differentiated zones. In the degenerative areas the myofibrills are disoriented and no distinct bands are visible. The mitochondria are filled with more or less connected fragments of cristae. Between the filaments elements of the sarcoplasmic reticulum and T-system are irregularly scattered. In the whole flight muscle of the newly emerged *Panorpa* the sarcoplasmic reticulum is very prominent as it is in muscles of 9 week and older imagos. Flight muscles of those of 17 days are characterized by a small but consistent area of sarcoplasmic reticulum and relatively few mitochondria compared with the portion of myofibrills. All structures appear fully differentiated and operable. After 54 days the first signs of beginning disorganization can be seen. Several mitochondria are enlarged as "giant mitochondria" probably by fusion, some of these are deformed and show whorl-like figures as the sign of partial destruction. The cristae seem to be dilated. The sarcoplasmic reticulum is always extended and zones of disarranged myofibrills arise.

In the 63-day old muscle the degenerative changes proceed and now comprise greater areas. The sarcoplasmic reticulum in general is extensively hypertrophied and shows irregular shaped vesicles. The cristae mitochondriales appear dilated and irregular Zones with severe degenerative changes of mitochondria, myofibrills and sarcoplasmic reticulum occur between others with no visible histological changes. It remains to be mentioned that these not insignificant muscle degenerations coincide with the mean life span of *Panorpa*. The 91-day old muscle is in a stage of overall degeneration. The mitochondria are deformed, partially fused and agglomerated at the *z*-bands. The inflated sarcoplasmic reticulum is irregularly located next to the also dissoluted fibrills and interposed are large vacuolized areas which are probably lipid inclusions. Table 1 shows the quantitative determination of sarcoplasmic reticulum enlargement.

TABLE 1. MORPHOMETRIC DETERMINATION OF THE PORTION OF SARCOPLASMIC RETICULUM IN FLIGHT MUSCLES OF *Panorpa* OF DIFFERENT AGES

Age (days)	1	17	54	63
Sections per unit area	4.9 ± 2.1	9.4 ± 1.8	7.9 ± 2.4	4.3 ± 1.4
No. of muscle sections	24	38	60	79

The number of cross sections of SR-tubules per unit area were counted after identical magnification of all muscle sections.

Fat body (Fig. 2 G + H)

As is often the case the fat body was difficult to cut because it was very rich in lipids (the fine structure is not very well preserved). Therefore only the major structural changes

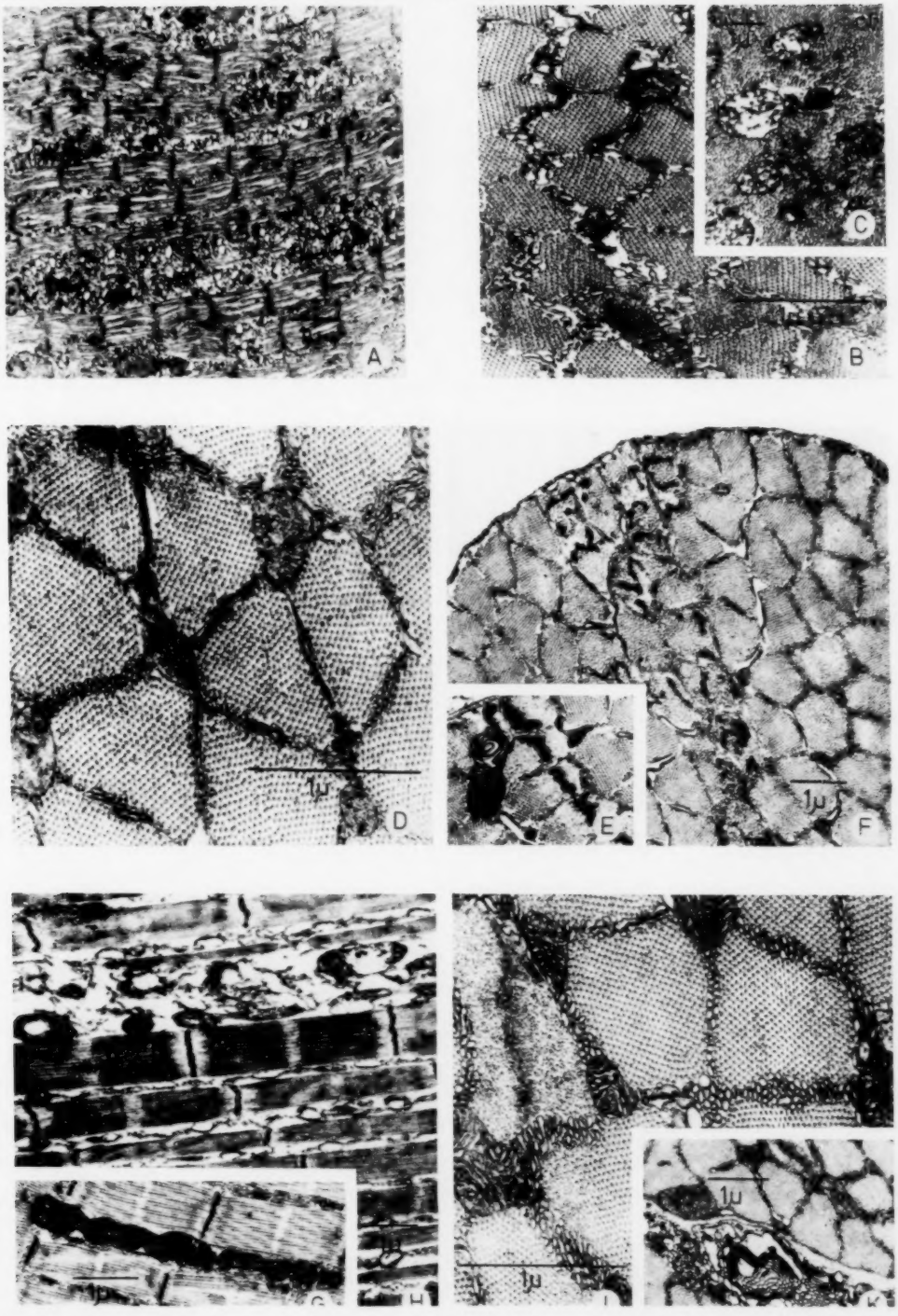

FIG. 1. (A–K) Flight muscle of *Panorpa*. (A–C) freshly emerged (one day). Longitudinal (A) and cross section (B). Bright areas which underwent dissolution lie between fully intact and differentiated zones. Mitochondria in the degenerative area with fragments of cristae (C). The sarcoplasmic reticulum and *T*-system are very prominent (D) 17 days (cross section). The portion of myofibrills is enlarged and surrounded by a small but consistent area of sarcoplasmic reticulum. All structures are well preserved. (E–K) 54 days. Focal areas of degeneration appear (middle region of F and left part of I in cross section and upper region of H in longitudinal section). Mitochondria are fused to "giant mitochondria" (G, E) and some are in different state of destruction (whorl-like figure in E, dissoluted areas in H). The sarcoplasmic reticulum is always extended (I) and membranes of *z*-system together with the sarcoplasmic reticulum are disarranged in focal areas (H).

Vol.
16
1981

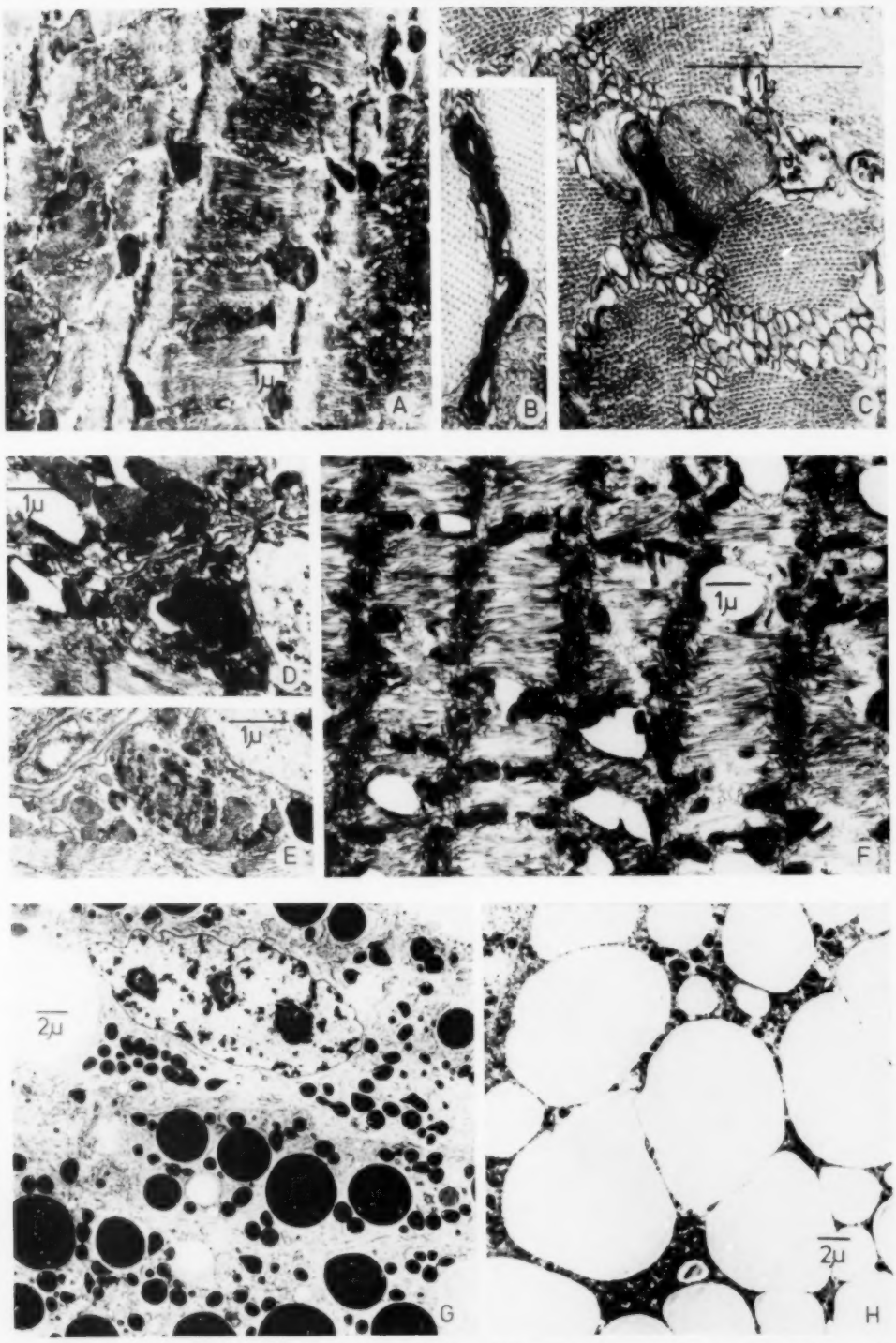

FIG. 2. (A–F) Flight muscle of *Panorpa*. (A–E) 63 days. The muscle degeneration proceeds and comprises larger areas (A). The mitochondria are largely deformed, others are destructed to form prominent electron dense residual bodies with membraneous structures (C–E). Membranes of *T*-system are collapsed (B). The sarcoplasmic reticulum is hypertrophied (C). (F) 91 days. A stage of overall degeneration (longitudinal section). Mitochondria are deformed and agglomerated at the *z*-bands. Myofibrills are totally dissoluted, the sarcoplasmic reticulum is inflated. Large vacuolized areas are interposed. (G–H) Fat body of *Panorpa*. (G) One day after emergence. Many strong osmiophilic inclusions (unsaturated lipids or proteins lie between several others which are poor osmiophilic (saturated lipids). Many mitochondria and parts of rough endoplasmic reticulum can also be seen. (H) 91 days. Fatty degeneration. The droplets with saturated lipids are very enlarged and tend to coalesce. All other structures are displaced.

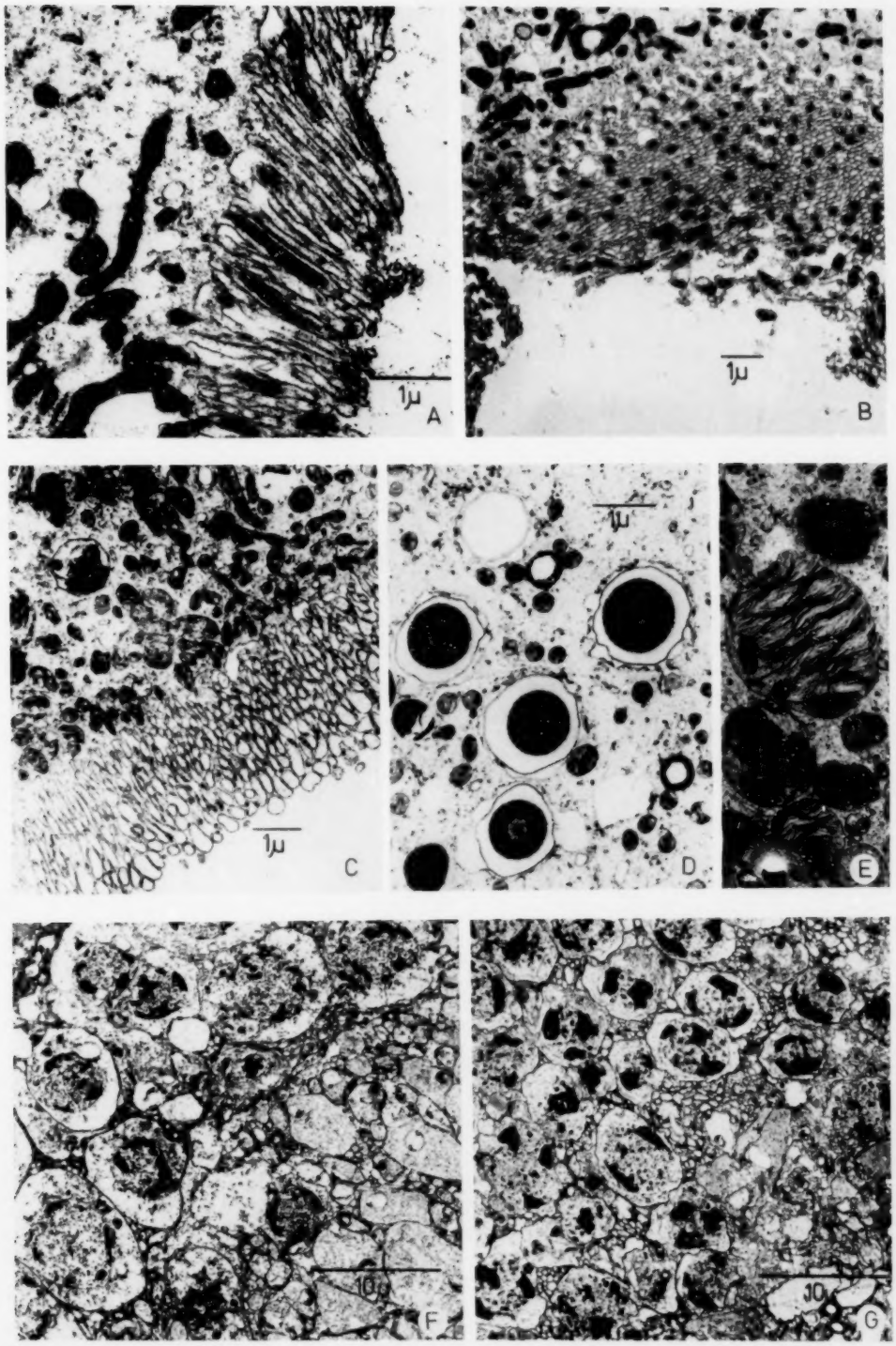

FIG. 3. (A–E) Malpighian tubule of *Panorpa*. (A–B) 3 days. Large mitochondria are located in the apical region, partly in the microvilli of the brush border (A: longitudinal, B: cross section). (C–E) 91 days. The brush border is completely free of mitochondria and appear to be swollen. Mitochondria at the base of the brush border are in different stages of dissolution (C). Abundant inclusions can be seen as the myeloid figures (C, E) or the renal-stone like sperules (D). (F–G) Brain (optic lobes) of *Panorpa*. (F) 27 days. Numerous neuron cells with prominent nuclei and wide perikaryon lie between axon branches and glial cell ramifications. (G) 79 days. The perikaryon is shrunken and the glial cell cytoplasm is reduced. The chromatin is more electron dense.

Vol.
16
1981

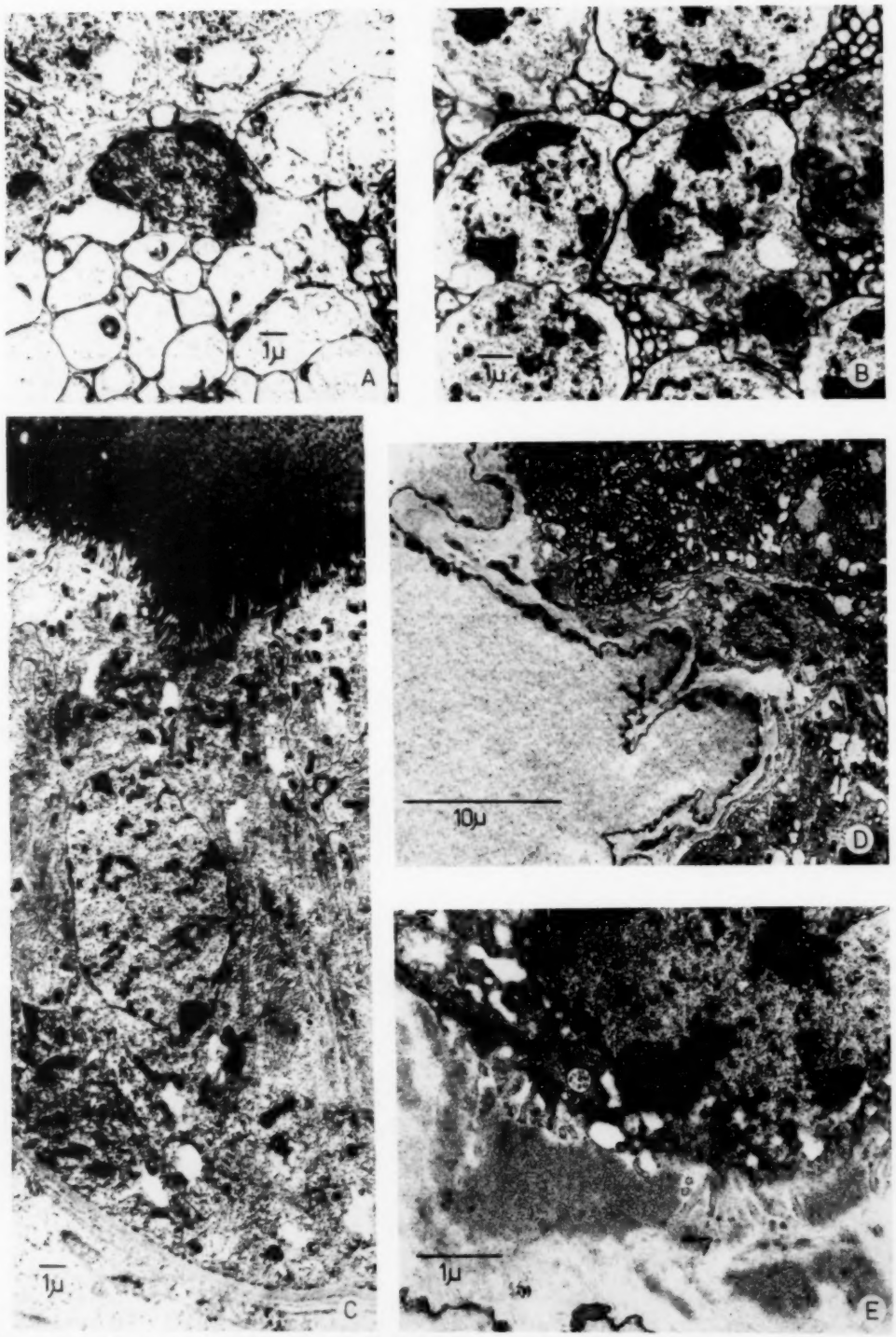

FIG. 4. (A–E) Brain (optic lobes) of *Panorpa*. (A) 27 days. Same region as in Fig. 3 (F) but with higher magnification. Glial cell membranes, axon branches and neuron cells are visible. (B) 79 days. Collapsed membranes of glial cells lie between neuron cells. A nucleus of a neuron cell is in stage of dissolution. Several residual bodies with myeloid structure are also present (middle right). (C–E) Salivary glands of *Panorpa* (C) 21 days. Typical appearance of a transport-epithel cell with a prominent apical brush border and deep basal infoldings. The mitochondria are located along the apical and basal side. A rough endoplasmic reticulum is extensively developed. The lumen is filled with distinct particles of a proteinaceous secretion. (D–E) 70 days. The brush border is vanished and no distinct particles can be seen in the lumen (D). A degenerating nucleus is shown in (E).

were regarded here. These changes cover the lipids itself. In the fat body of 1-day old imagos many strong osmiophilic and several poor osmiophilic inclusions are distributed. It is assumed that both these inclusions are lipids, the strong osmiophilic being unsaturated ones. In the fat body of 91-day old individuals the saturated lipid droplets are very enlarged and tend to coalesce. They displace all the other structures so that the old fat body undergoes a fatty or cirrhotic degeneration.

Malpighian tubules (Fig. 3 A–E)

Malpighian tubules from 3- and 91-day old adults were compared. The tubules of the young adults have the typical appearance of transport epithelia with an extensively developed basal labyrinth on the basal side and a brush border on the apical side. Large mitochondria are located apically (like in a number of other insect species) in the microvilli and basal near the infoldings. Looking at the tubules of the old adults the mitochondria are completely disappeared from the brush border lying now on its base. The brush border itself appears to be swollen and the mitochondria are in different stages of lysis. Involved in this process are autophagic vacuoles. The cytoplasm of the tubulus cells is filled with abundant spherical inclusions like renal stones. Finally numerous myeloid figures and other residual bodies packed with electron-dense material (lipofuscin) are visible. All these vesicles are surrounded with a membrane and therefore were assumed to be of lysosomal origin.

Vol. 16 1981

Salivary glands (Fig. 4 C–E)

Freshly emerged, 21- and 70-day old males were studied. Male *Panorpae* are distinguished by well developed salivary glands which extend up to the abdomen and produce a proteinaceous secretion, a so called "love candy", during the courtship behaviour. As with malpighian tubules, these glands represent a typical transport epithelium. The extensive rough endoplasmic reticulum points to the protein synthesizing function. The lumina of the salivary glands of freshly emerged and 21-day old adults are closely packed with the secreted material, visible as distinct particles. Up to this time the fine structure of the glands is well preserved. In the glands of 70-day old males, however, the protein particles are absent, the brush border is vanished and degenerating nuclei can be seen. The rough endoplasmic reticulum is reduced.

Brain (optic lobes) Fig. 3 F + G (Fig. 4 A + B)

For the examination of histological changes in the brain, the optic lobes especially in the region of the neuropile were chosen and compared in 27- and 79-day old males.

The neuropile of the young adults shows numerous neuron cell bodies with prominent nuclei, surrounded by a wide perikaryon together with many axon branches. Glial cytoplasm and membranes run in parallel strands between neuron cell bodies and axons. The most striking difference in the aged brain involves the reduction of the perikaryon mass and similarly the glial cytoplasm. The membranes of the glial cells are therefore collapsed. The chromatin of the nuclei appears to be more electron dense. Several residual bodies in the form of myeloid figures can be seen. Scattered autophagic vacuoles are also present. In contrast to the structural changes of the neuropile in the optic lobes no changes were detectable comparing the ommatidia in the composed eyes of young and old individuals. The cornea as well as the crystalline cones and the retinular cells with the rhabdom are well preserved just as the cell components itself.

DISCUSSION

The aim of the present study was to give a survey of histological changes with age and to extend such studies beyond dipterans and hymenopterans. It is therefore possible that minor changes especially of quantitative origin were not fully counted. It seems further desirable to give attention to other parts of the brain and to gonads and gut.

No ultrastructure study exists on wing ageing, a process which is so impressive in both dipterans and *Panorpa*. In *Panorpa* the break-down of the wings is accompanied by an accumulation of brown pigment (probably lipofuscin).

Despite this incompleteness the results show that—with the exception of the compound eyes—in all studied organs drastic ultrastructural degenerations take place with increasing age.

Time course of muscle degeneration

Most remarkable of the newly emerged adults is the full flight ability despite the massive focal degenerations in the region of the flight muscles, which obviously results from the break-down of the larval muscle. Immediately after emergence and up to one week of adult life the highest flight activities can be observed, decreasing thereafter. Similar in adults of 9 weeks, at the time of mean life span, the age correlated muscle dystrophy is in a progressive state but does not prevent the flying capacity after stimulation. Also the mating behavior which is accompanied by well coordinated wing movements lasts up to this time. The spontaneous flight, however, is very reduced at this age, demonstrating that the flight ability is not directly correlated with muscle degeneration. Only 13-week old individuals are nearly incapable of flying. As mentioned above, observations on nature and degree of age dependent muscle degeneration of other insects are often contradictory and this may be the result of different holding conditions of the same object (Finlayson, 1975; Turturro and Shafiq, 1979). An effect of diet on the ultrastructure of a hymenopteran flight muscle was also shown (Davies, 1974). In sharp contrast to Sohal (1976), Turturro and Shafiq (1979) found no appreciable changes in mitochondria and myofibrills of *Musca* after rearing under "optimal conditions".

Our own studies on *Phormia terrae novae* point in the same directions: 5- and 50-day old flight muscles of *Phormia* are—with the exception of "giant mitochondria" of the latter—nearly identical in their histological picture. Therefore, one must resume that clear species-specific differences in the histological expression of ageing exist.

A more common feature seems to be the increase in the amount of sarcoplasmic reticulum which was described from *Musca* (Turturro and Shafiq, 1979) and *Hymenoptera* (Davies, 1974). Due to the synchronous flight muscle type of *Panorpa* the increase of the sarcoplasmic reticulum area is much more prominent and essential in regard to the muscle function. The hypertrophy is an obvious adaptation to a poor substrate supply (calcium and others).

The picture resembles that of heart muscle sections of rats with an artificially induced left ventricular myocard hypertrophy (Wendt-Gallitelli and Jacob, 1977) and similar changes of extensor digitorum longus muscle in senile rats (Gutmann, 1977). Comparable in a functional manner is the described increase of the smooth endoplasmic reticulum in livers of old aged rats (Grinna, 1977).

Fat body

The extensive degenerative changes in the fat body of *Panorpa* which indicate a total loss of function of this organ with central metabolic activity are also not common to all

insects studied. The fat body of young imagos is already rich in lipids in contrast to *Musca* or other dipterans (Miquel *et al.*, 1972; Sohal, 1973; Thomsen and Thomsen, 1978). Glycogen, however, prominent in dipteran fat bodies, could not be detected either in young or in old fat bodies of *Panorpa*. This corresponds with the generally low carbohydrate levels in the body composition throughout the whole life (Collatz, unpublished).

An increase of lipids in old fat bodies of *Musca* was described (Sohal, 1973) but this increase by no means reaches that extent of *Panorpa*. Our own observations on *Phormia* show in contrast an overall reduction of lipid vacuoles in old fat bodies. In *Drosophila* the picture is not clear. Both a drastic shrinkage and a great lipid depot in fat bodies of old age subjects have been reported (Rockstein and Miquel, 1973). The strong osmiophilic droplets in the fat body of one day old imagines are assumed to be unsaturated lipids. However, the possibility that they consist of protein cannot yet be ruled out. Preliminary gas–chromatographic analyses support the assumption of a change in the lipid composition toward more saturated fatty acids in the older imagines. It might be possible that the more dominant, saturated long chain fatty acids are more difficult to metabolize. Moreover, the fatty acid compostion of old free living *Panorpae* correspond to that of the laboratory reared specimens so that a feeding effect on fatty acid composition can be excluded. In summary the fatty degeneration of the fat body of *Panorpa* appears to such an extent that minor changes are not visible.

Vol. 16 1981

Residual bodies in the malpighian tubules

Myeloid figures and other types of residual bodies which are abundant in the ageing malpighian tubules—but also visible in other organs of *Panorpa*—are meanwhile so often described in various organisms that they appear to be a typical ageing design. The nature and interconversion of the bodies were discussed (Sohal and Sharma, 1972; Miquel *et al.*, 1972) and may not be further dealt with here. They are not, however, age specific but appear generally in degenerating processes. The spherical renal stone-like inclusions are to be distinguished from the other residual bodies. They are known from several other insects but lie more often in the lumen of the malpighian tubules (Wessing and Eichelberg, 1975). Abundant as both of these inclusions in the transport epithelia are, they may have a severe disturbing effect on its function and therefore on the homeostasis of the whole insect.

Salivary glands

The salivary glands give us an example of a highly specific loss of function. Its histological changes influence directly the behavior of *Panorpa* and lead to a reduction of the frequency of courtship behavior with increasing age. No comparable data exist and it would be of interest to correlate the quantitative analysis of mating behaviour with the degenerative changes of the male salivary glands.

Nervous system

The age dependent reduction of the cytoplasmic volume of the neuron cells seems to be a common feature in mammals (Bondareff, 1964, 1975) and was also described from *Drosophila* (Miquel, 1971; Herman *et al.*, 1971). However, only scarce knowledge exists on the ageing of the insect nervous system. Focal cytoplasmic degenerations with autophagic vacuoles and the accumulation of residual bodies in the brain of *Phormia* were

described (Sohal and Sharma, 1972). Their pictures are readily comparable with those of *Panorpa*. Furthermore, the authors report a reduction of ribosomes with age, which we have not seen in *Panorpa*. Also regarding the reduction of glial cytoplasm, we can assume that a poor metabolic supply of the neuron cells may exist at old age.

SUMMARY

1. Age related ultrastructural changes in flight muscle, fat body, malpighian tubules, salivary glands, and optic lobes of male mecopteran flies *Panorpa vulgaris* were studied.

2. In all these organs striking degenerative changes arise with increasing age. Flight muscle degeneration is characterized by myofibrill dissolution, deformed and "whorl-like" mitochondria and an extensive hypertrophy of sarcoplasmic reticulum which cover the whole muscle in 13-week old adults. In fat body of aged adults a fatty "cirrhotic degeneration" takes place and a change in fatty acid composition of lipids is assumed. Malpighian tubules in 91-day old individuals show abundant inclusions of the myeloid-type and renal stone-like spheric residual bodies. The mitochondria completely disappeared from the brush border and exhibit different stages of degeneration. The salivary glands of old males lack the protein granules lying in the lumen of the young glands. In the optic lobes (neuropile area) glial and neuronal cytoplasm become reduced with advanced age. Scattered autophagic vacuoles and residual bodies are also present. In contrast, no changes in the optic apparatus itself could be detected.

3. The results were compared with those of *Phormia terrae novae,* where no such degenerative changes occur.

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Vol.
16
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AGE-RELATED CHANGES IN HEMOPOIETIC MICROENVIRONMENT. ENHANCED GROWTH OF HEMOPOIETIC STROMA AND WEAKENED GENETIC RESISTANCE TO HEMOPOIETIC CELLS IN OLD MICE

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Vol.
16
1981

INTRODUCTION

THE CAUSES and mechanisms of the ageing of various cell systems are not known. Cell renewal populations, the hemopoietic system in particular, have been studied most in this respect (Kay and Makinodan, 1976). It was shown that with age the total number of hemopoietic stem cells in the bone marrow remains constant or even increases slightly (Chen, 1971; Coggle *et al.*, 1975). Young and old stem cells are equally capable of repopulating stem cell-deficient mice (Harrison, 1975; Lajtha and Schofield, 1971; Micklem *et al.*, 1973; Ogden and Micklem, 1976), however, the growth capacity of stem cells declines somewhat with age (Albright and Makinodan, 1976). One line of hemopoietic stem cell differentiation, the T lymphocytes, suffers most, evidently due to the age involution of the stroma of the thymus, the central lymphoid organ which is necessary for the differentiation of T lymphocytes (Hirokawa and Makinodan, 1975).

That is why the problem of age-related changes in the stroma of other hemopoietic organs arose, especially as environmental deficiencies were revealed in old animals with regard to the ability to maintain the growth of hemopoietic colonies in the spleen (Vacek *et al.*, 1976) and the rate of growth of the antibody-forming cell population in it, though only about 10 per cent of normal age-related decline can be attributed to changes in the cellular milieu (Price and Makinodan, 1972).

In the present work the problem of the age-related changes in the bone marrow microenvironment was studied on models of ectopic bone marrow transplantation and hybrid resistance to hemopoietic cells expressed in poor growth of the parental hemopoietic cells in irradiated FI hybrids of some mouse strains (for references see Chertkov and Friedenstein, 1977).

METHODS AND PROCEDURE

Female C57B1/6 and (C57B1/6 × CBA/H) FI (further B6CFI) mice were used. The femoral bone marrow plugs were implanted beneath the renal capsule of anaesthetized recipients. One month later the size of the hemopoietic microenvironment formed was estimated by counting the number of hemopoietic cells in the focus of ectopic hemopoiesis. The size of the focus is proportionate to the number of stromal precursors capable of transferring the hemopoietic microenvironment (Chertkov and Gurevitch, 1977). Cells from 8–10 foci were pooled for each point. The number of hemopoietic stem cells (CFUs) in the bone marrow, spleen, ectopic hemopoietic focus was determined by the method of Till and McCulloch (1961) in mice irradiated with ^{137}Cs gamma-rays in a dose of 1300 rads (B6CFI) or 1100 rads (C57B1/6). The animals were killed 8 days after the injection of hemopoietic cells (3–5 × 10^4 bone marrow cells or 3–5 × 10^5 spleen cells) and the number of macroscopic colonies was counted. To determine the kinetics of CFUs growth in the spleen of irradiated mice, 2 × 10^6 bone marrow cells were transplanted to intermediate recipients irradiated with 1100–1300 rads and the content of CFUs in their spleens was determined in 7 and 10 days by transplanting cells in

a dose of 1/5–1/250 spleen equivalent to irradiated recipients syngeneic with the donor of the initial bone marrow. The cellularity of the various hemopoietic organs and the myelogram were determined by the routine methods. The intensity of erythropoiesis was estimated according to the incorporation of ^{59}Fe for 4 h after its intravenous infusion in the form of ferric ascorbate in a dose of 0.5 μCi/mouse and expressed in per cent incorporation per total injected activity.

RESULTS

The lifespan of B6CF1 mice is approximately 30 months. Hemopoiesis in these mice at the age of 2 yr did not differ from that in young animals in any of the values studied (Table 1), the myelogram (data not shown) was also in accord with the initial one. The number of stromal precursors, which was judged according to the size of the focus of ectopic hemopoiesis formed by them in standard recipients (mice 8–12 weeks of age), did not change in the 2-yr lifespan of the mouse (Table 2). At the same time, the growth of the ectopic focus of hemopoiesis in old mice proved to be intensified. Significant differences in the size of the ectopic focus are detected for the first time at the age of 1 yr, when the focus in such recipient grows to about twice the size of that in the standard recipient. The size of the focus in old mice at the age of 2 yr is approximately three times that in the standard recipient irrespective of whether old or young bone marrow is transplanted (Table 2).

Hemopoiesis in ectopic foci in old and young recipients following implantation of old and young bone marrow was similar in quality and did not differ from that in normal bone marrow. The CFUs content and the intensity of ^{59}Fe incorporation were in accordance with the cellularity of the focus.

Thus, old mice possess factors stimulating growth of the implanted bone marrow stroma. Similar stimulation is also induced by total body irradiation of the recipient (Chamberlin *et al.*, 1974; Chertkov and Gurevitch, 1977), which, in addition, weakens the hybrid resistance to hemopoietic cells considerably (Lemeneva and Chertkov, 1974). In view of this, we studied the hybrid resistance of old B6CF1 mice to bone marrow of the C57BL/6 genotype. At the age of 2 yr hybrid resistance proved to be sharply diminished. On injecting the same bone marrow suspension into old and young mice, the number of hemopoietic colonies in old mice was 3.5 times that in the young (Table 3). The kinetics of CFUs growth in the spleen of young and old mice were also in accord with this: both 7 and 10 days after transplantation of parental bone marrow cells to hybrids the CFUs content in the spleen of old recipients was almost 3 times that in young recipients. In the same time, as it is shown in Table 3, old and young mice were equally capable of maintaining the growth of syngeneic hemopoietic cells in the spleen.

DISCUSSION

The results obtained show that the content of cells in the bone marrow which are capable of transferring the hemopoietic microenvironment does not diminish with age. However, factors stimulating the growth of implanted stromal precursors were revealed in old animals; on the other hand, the hybrid resistance to hemopoietic graft in old animals is greatly weakened. Since the stroma of the hemopoietic organs is responsible for the resistance to hemopoietic cells (Chertkov and Gurevitch, 1980) it therefore may be assumed that the stroma of old animals is incapable of accomplishing at least some of the functions necessary for the control of cell proliferation in the hemopoietic system. Study of the possible significance of this phenomenon in the increase in the incidence of malignant diseases in old animals is of obvious interest.

Vol. 16 1981

TABLE 1. HEMOPOIESIS IN OLD MICE

Age	Femur				Spleen			
	Cellularity ($\times 10^{-6}$)	CFUs/10^5 cells	CFUs/femur	^{59}Fe uptake per femur	Cellularity (10^{-6})	CFUs/10^5 cells	CFUs/spleen	^{59}Fe/uptake per spleen
2 months	21.1 ± 1.7	25.7 ± 3.3	5400 ± 1134	1.5% ± 0.12	410 ± 44	0.9 ± 0.3	3700 ± 903	3.0% ± 0.29
2 yr	24.0 ± 1.9	18.3 ± 2.0	4400 ± 836	1.3% ± 0.11	460 ± 51	1.1 ± 0.2	4900 ± 1421	3.9% ± 0.40

TABLE 2. STROMAL PRECURSORS AND HEMOPOIESIS IN ECTOPIC HEMOPOIETIC FOCI IN OLD MICE

Donor age	Recipient age	Cellularity of focus ($\times 10^{-6}$)	CFUs/10^5 cells	CFUs/focus	^{59}Fe uptake/focus
2 months	2 months	10.7 ± 1.1	13.5 ± 1.5	1990 ± 396	1.08% ± 0.11
6 months	2 months	8.0 ± 0.9 (NS)	31.2 ± 1.6 ($p < 0.001$)	2500 ± 400 (NS)	—
1 yr	2 months	10.8 ± 1.6 (NS)	—	—	—
2 yr	2 months	9.0 ± 0.9 (NS)	17.5 ± 2.5 (NS)	2100 ± 358 (NS)	0.73% ± 0.08 (NS)
2 months	6 months	12.6 ± 1.4 (NS)	13.5 ± 5.9 (NS)	1701 ± 921 (NS)	—
2 months	1 yr	20.3 ± 2.4 ($p < 0.05$)	—	—	—
2 months	2 yr	27.1 ± 2.6 ($p < 0.001$)	13.5 ± 1.0 (NS)	4900 ± 333 ($p < 0.05$)	1.80% ± 0.21 ($p < 0.01$)
2 yr	2 yr	31.9 ± 3.2 ($p < 0.001$)	15.8 ± 1.4 (NS)	6700 ± 1610 ($p < 0.001$)	2.0% ± 0.19 ($p < 0.001$)

TABLE 3. HYBRID RESISTANCE TO HEMOPOIETIC GRAFT IN OLD MICE

Bone marrow donor	Recipient and his age	Colonies/10^5 cells	CFUs/spleen after injection of 2×10^6 C57B1/6 bone marrow cells: after 7 days		after 10 days	
B6CF1	B6CF1, 2 months	24.9 ± 1.3	1210 ± 235			
C57B1/6	C57B1/6, 2 months	15.7 ± 1.9	1025 ± 224			
C57B1/6	B6CF1, 2 months	2.6 ± 1.4	140 ± 23	$(p < 0.001)$	1730 ± 263	$(p < 0.001)$
C57B1/6	B6CF1, 2 yr	8.9 ± 1.4	388 ± 25		4100 ± 756	

SUMMARY

The growth of transplanted hemopoietic stroma in mice aged 1 yr and older is intensified, as the result of which the focus of ectopic hemopoiesis formed in transplantation of a bone marrow fragment is twice to 3 times the size of that in young animals. In 2-yr old (C57BL/6 × CBA/H) F1 mice genetic resistance to C57BL/6 hemopoietic cells is greatly reduced.

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LOW LEVEL OF U.V.-INDUCED UNSCHEDULED DNA SYNTHESIS IN POSTMITOTIC BRAIN CELLS OF HAMSTERS: POSSIBLE RELEVANCE TO AGING

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Vol. 16 1981

INTRODUCTION

SEVERAL observations indicate that DNA excision repair is deficient in postmitotic cells. The altered bases, O^6-methyl- and O^6-ethylguanine, are excised *in vivo* from the postmitotic rat brain at a much slower rate than from rat liver or kidney (Goth and Rajewsky, 1974; Margison and Kleihues, 1975). Between 6 and 15 days of embryogenesis, chick neural retina cells undergo an approx. 10-fold reduction in the rate of DNA excision repair induced by methyl methanesulfonate (MMS) or N-acetoxy acetylaminofluorene (AAF, Karran *et al.*, 1977). In addition, the differentiation *in vitro* of chick embryonic pectoral myocytes to the postmitotic state is accompanied by a 2-fold reduction in u.v.-induced unscheduled DNA synthesis (UDS) (Stockdale, 1971). Excision repair was evident in primary heart muscle cell cultures from neonatal rats, but not in the postmitotic heart fibres from young adult rats (Lampidis and Schaiberger, 1975).

Consideration of the important coordinating functions of the brain (i.e. to receive and analyze sensory stimuli, to control muscular movement and to regulate metabolism through the endocrine system) led us to study the repair capacity of the mitotic and postmitotic hamster brain and to relate this capacity to aging. For comparison, we also measured u.v.-induced UDS at various ages in the hamster liver, as well as in the lung and kidney (see companion paper, Gensler, 1981).

The use of freshly isolated brain cells, with a relatively rapid exposure to u.v. radiation and to tritiated thymidine, obviated the need for culturing embryonic neurons. We report here that brain cells have a low rate of u.v.-induced UDS. In addition, hamster liver cells may also have a low rate of repair but the measurement is complicated by an inhibitor(s) or repair which is released when liver tissue is minced. The low levels of repair in the brain and liver of the young adult hamsters are maintained through at least ⅔ of the maximum life span of the animal. However, the level of u.v.-induced UDS is approx. 3-fold higher in early mitotic brain cells than in postmitotic brain cells.

MATERIALS AND METHODS

The methods used are described in the companion paper (Gensler, 1981). However, due to release of repair inhibitors during mincing, the brain and liver were minced into a larger volume of suspending medium, at a ratio of 1 g of tissue to 100 ml Hank's Balanced Salt Solution (HBSS). Debris in brain tissue suspensions was removed by centrifuging at 20 ***g*** for 3 min and discarding the precipitate. Cells in liver and brain suspensions were concentrated by centrifuging at 730 ***g*** for 10 min and discarding the supernatant. The cells were then adjusted to a concentration of 5×10^6 cells/ml in HBSS for u.v. irradiation.

RESULTS

Brain

Table 1 shows the levels of DNA synthesis in u.v.-irradiated and unirradiated brain cells isolated from hamsters of various ages. The percent of irradiated brain cells which underwent repair averaged 13.3 at days 4–8, but decreased to 2.6 by day 39 and fluctuated around a mean of 5.4 throughout the age span studied. Few (2.5% or less) unirradiated brain cells showed UDS. Repair synthesis, which we have defined as the product obtained by multiplying the percent of cells showing repair by the mean grain count per repairing cell, averaged 179 in irradiated brain cells of 4–8 day old hamsters, and then dropped to fluctuate around a mean of 53 thereafter (Table 1). Repair synthesis in unirradiated brain cells averaged 11.7 at 4–8 days, but averaged about 5.8 during the remainder of the ages studied. The percent of irradiated and unirradiated cells in S phase dropped from approx. 6.6 during days 4–8 to a mean of 0.4 during days 39–551.

TABLE 1. UNSCHEDULED DNA SYNTHESIS IN U.V.-IRRADIATED AND UNIRRADIATED BRAIN CELLS ISOLATED FROM HAMSTERS OF VARIOUS AGES

u.v. radiation	Age (days)	Mean grain count*	S.E.	Cells with UDS No.	Cells with UDS % of total cells	Repair synthesis†	Cells in S phase (%)	Viability (%)
+	4	13.7	0.94	107	15.5	213	8.3	86
−	4	7.3	0.96	9	1.3	9.2	6.6	
+	5	13.7	0.85	128	6.5	89.1	7.3	85
−	5	6.8	0.97	5	0.6	4.2	7.5	
+	8	13.0	0.65	189	18.0	234	7.2	80
−	8	14.5	3.04	15	1.5	21.8	2.9	
+	39	15.1	1.79	28	2.6	39.6	0.2	85
−	39	6.7	0.33	3	0.3	1.9	0.1	
+	56	7.9	0.61	46	6.1	47.7	0.4	81
−	56	8.6	2.71	5	0.5	4.0	0.5	
+	57	10.8	0.65	51	3.8	41.6	0.1	85
−	57	5.5	0.50	2	0.2	0.9	0.2	
+	125	10.8	0.82	57	5.3	48.2	0.7	87
−	125	6.4	0.43	7	1.1	6.9	0.6	
+	129	9.0	0.67	37	3.3	29.4	0.4	91
−	129	9.6	0.94	21	2.2	20.9	0.4	
+	133	9.9	0.38	192	10.5	104	0.6	92
−	133	6.5	0.42	18	2.4	16	0.2	
+	281	10.1	0.60	96	7.4	74.3	1.6	95
−	281	7.8	0.87	6	0.5	3.9	0.7	
+	283	10.3	0.58	65	4.3	44.6	0.1	92
−	283	12.3	3.76	3	0.2	2.9	0.5	
+	300	8.4	0.83	25	3.6	30.5	0.4	99
−	300	6.3	0.42	7	0.8	4.8	0.1	
+	534	8.8	0.49	71	7.6	66.9	0.7	95
−	534	9.2	3.25	5	0.5	4.3	0.8	
+	547	9.4	0.47	133	6.8	63.7	0.4	93
−	547	10.0	0	1	0.1	0.6	0.1	
+	551	11.5	0.92	60	4.0	45.5	0.7	86
−	551	11.7	6.17	3	0.2	2.6	0.4	

*The mean grain count is of nuclei undergoing unscheduled DNA synthesis, i.e. displaying 5–55 grains.

†Repair synthesis is the product of the mean grain count multiplied by the percent of total cells exhibiting UDS.

Figure 1 depicts the mean values obtained from Table 1 by grouping the data in clusters of three individual hamsters of similar ages. Figure 1a shows the early drop in percent of irradiated brain cells undergoing UDS, and the subsequent low level of repairing cells. In Fig. 1b the mean grain count can be seen to remain relatively constant from day 4 to 551. The early decrease and subsequent low levels of the proportion of brain cells in S phase is shown in Fig. 1c.

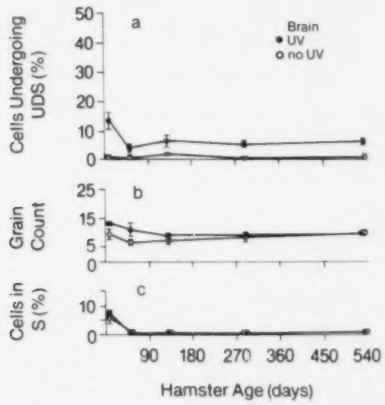

FIG. 1. The influence of hamster age on DNA synthesis in u.v.-irradiated and unirradiated brain cells. Mean values ± S.E. obtained with three individual hamsters of similar age are plotted. ●, irradiated cells; ○, unirradiated cells. (a) Frequency of brain nuclei showing u.v.-induced UDS at different ages. (b) The mean grain count of brain nuclei displaying UDS at different ages. (c) Frequency of brain nuclei in S phase at different ages.

Figure 2 displays the early reduction in repair synthesis in irradiated brain cells, followed by a relatively constant low level of repair. The mean value for repair synthesis is 53 in postmitotic brain cells, 553 in adult lung cells and 237 in adult kidney cells (Gensler, 1981).

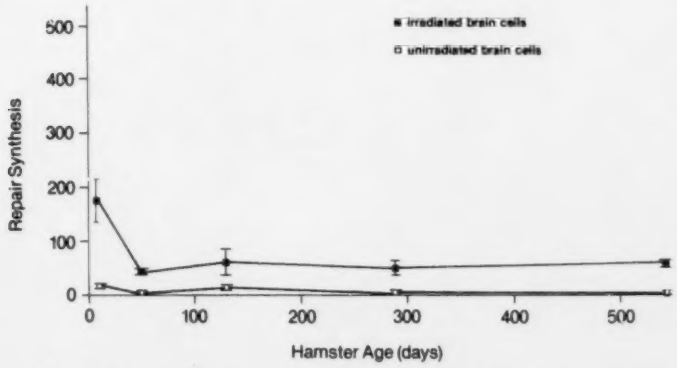

FIG. 2. Repair synthesis in u.v.-irradiated and unirradiated hamster brain cells at various ages. Mean levels ± S.E. of repair synthesis obtained with three individual hamsters of similar age are plotted. Repair synthesis is the mean grain count over 100 nuclei, obtained by multiplying the percent of nuclei undergoing UDS by the mean grain count per repairing nucleus. ●, irradiated nuclei; ○, unirradiated nuclei.

Vol. 16 1981

Liver

Table 2 presents the levels of DNA synthesis in u.v.-irradiated and unirradiated liver cells isolated from hamsters of various ages. An average of 14% of irradiated liver cells underwent UDS at days 8 and 9, but after day 38 approx. 6% were repairing. However, this is probably an underestimate of the number of liver cells capable of excision repair, as discussed below. Repair synthesis was approx. 3-fold higher at days 8 and 9 than after day 39, although this may reflect a bias because of a single high value at day 9. Irradiated liver cells had 3- to 30-fold more repair synthesis (average 16-fold) than did unirradiated cells. The percent of irradiated and unirradiated liver cells in S phase decreased from an average of 5.1 on days 8 and 9 to an average of 0.8 on days 45 and thereafter.

TABLE 2. UNSCHEDULED DNA SYNTHESIS IN U.V.-IRRADIATED AND UNIRRADIATED LIVER CELLS ISOLATED FROM HAMSTERS OF VARIOUS AGES

u.v. radiation	Age (days)	Mean grain count*	S.E.	Cells with UDS No.	Cells with UDS % of total cells	Repair synthesis†	Cells in S phase (%)	Viability (%)
+	8	8.4	0.35	152	10.7	90.0	5.0	95
−	8	9.1	0.59	22	1.3	10.4	3.7	
+	9	15.6	0.63	177	31.0	484	8.1	96
−	9	8.8	2.51	6	1.0	9.1	7.8	
+	9	8.4	0.36	114	7.9	66.5	5.4	99
−	9	6.3	0.75	4	0.3	2.2	6.9	
+	9	8.6	0.30	111	6.6	56.8	2.5	94
−	9	6.5	0.43	6	0.6	3.9	1.0	
+	39	11.5	1.60	20	2.0	22.5	4.4	99
−	39	6.3	1.33	3	0.3	2.4	3.0	
+	45	9.5	0.33	146	8.8	83.7	0.6	96
−	45	5.7	0.33	6	0.4	2.4	0.8	
+	45	9.3	0.65	69	4.7	43.3	1.8	96
−	45	6.0	0.45	5	0.4	2.1	0.9	
+	122	9.4	0.54	64	6.0	56.9	0.6	85
−	122	5.8	0.40	8	0.4	2.5	0.2	
+	125	7.7	0.40	47	6.76	51.9	0.9	85
−	125	6.7	0.78	7	0.96	6.4	0.7	
+	129	9.2	0.49	87	7.38	68.2	1.0	88
−	129	6.5	0.63	8	0.96	6.2	0.8	
+	281	12.4	0.77	43	6.24	77.6	0.9	86
−	281	11.3	2.64	12	2.59	29.3	1.1	
+	283	8.7	0.47	50	4.03	34.9	0.6	89
−	283	6.6	0.60	9	0.65	4.3	1.1	
+	300	6.6	0.39	11	1.23	8.1	1.2	80
−	300	7.6	1.04	7	0.70	5.3	1.0	
+	421	8.3	0.37	99	9.03	74.7	0.4	81
−	421	5.0	0	1	0.09	0.4	0.8	
+	428	6.9	0.227	66	5.07	35.1	0.6	82
−	428	6.3	0.518	10	0.72	4.5	0.7	
+	428	6.9	0.227	80	4.60	31.6	0.5	84
−	428	5.9	0.512	9	0.67	3.9	0.5	

*See footnote to Table 1.
†See footnote to Table 1.

Figures 3 and 4 summarize the experiments with liver cells. Each point represents the mean value of 3 or 4 individual hamsters of similar age taken from Table 2. Figure 3a shows that the percent of cells undergoing repair on day 9 was accompanied by a large

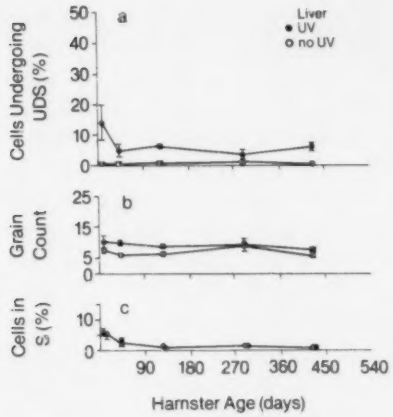

FIG. 3. DNA synthesis in u.v.-irradiated and unirradiated liver cells at different hamster ages. The legend is similar to that of Fig. 1.

Vol. 16 1981

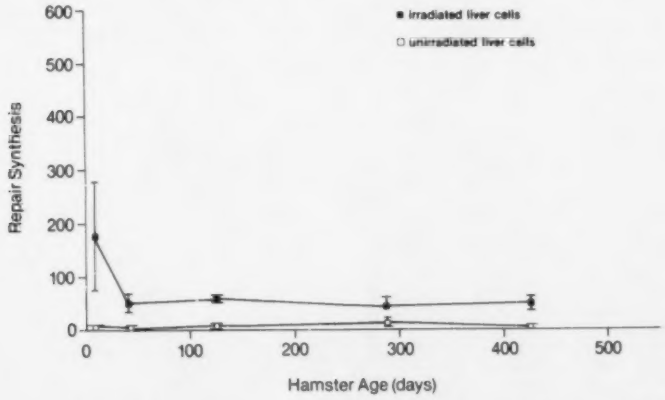

FIG. 4. Repair synthesis in u.v.-irradiated and unirradiated liver cells at different hamster ages. The legend is similar to that of Fig. 2.

enough standard error so that the early decrease in number of repairing irradiated cells may not be significant. Between days 42 and 426, the percent of irradiated cells showing UDS remained approximately constant. The mean number of grains per repairing liver cell was similar throughout the age span studied, as seen in Fig. 3b. The percent of liver cells in S phase dropped as the animal reached adulthood and remained low at least to day 426 (Fig. 3c). Repair synthesis of liver cells is plotted in Fig. 4. The early decrease between day 9 and 42 may not be significant, due to the large standard error of day 9. Adult irradiated liver cells maintained a relatively constant low level of repair synthesis to day 428.

Inhibition assay

The tissue level of UDS, measured *in vitro* in cell suspensions, could be influenced if tissue mincing releases excision repair inhibitors into the medium. Proteases have previously

been found to interfere with determinations of immunoglobulin production in liver cell suspensions prepared by tissue mincing (Melchers *et al.*, 1975). To detect an inhibitor, the supernatant fluid from tissue minces and 10-fold dilutions of the fluid were used to suspend lung cells, and the levels of u.v.-induced UDS of the cells in these supernatants were compared with the UDS level of lung cells suspended in HBSS. Lung cells were chosen for this inhibition assay because their viability was always high and they had the greatest level of u.v.-induced UDS, thereby affording a sensitive detection system. Figure 5 shows the percent of inhibition of the number of lung cells undergoing repair after exposure to supernatants of liver, brain, kidney or lung tissues. These tissues were minced at a concentration of 0.4 g in 4.0 ml or 40 ml of HBSS. The kidney and lung supernatants did not inhibit lung cell repair at either concentration. The brain supernatant from the more concentrated tissue mince inhibited 43% of the lung cells from repairing, whereas no inhibition occurred in the more dilute supernatant. This inhibition was evident without any cytotoxicity as detected by trypan blue dye exclusion. The dilute and concentrated liver supernatants inhibited 49 and 90% of the lung cells, respectively, from performing UDS. In additional experiments the concentrated liver supernatant also inhibited 99% of irradiated kidney cells from undergoing repair, so that kidney cells responded to liver supernatant as did lung cells. Inhibition was manifested by a reduction in the number of cells showing repair, rather than reduction in the mean grain count of repairing cells, as if the cells were turned off completely or not at all.

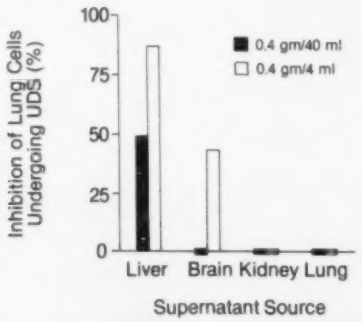

FIG. 5. The extent of inhibition of u.v.-induced UDS in lung cells by supernatants from selected tissue minces. ■, cells exposed to supernatant from 0.4 g tissue minced in 40 ml medium; □, cells exposed to supernatant from 0.4 g tissue minced in 4.0 ml medium.

Mincing tissues at a higher dilution than 0.4 g/40 ml resulted in excessive cell loss, therefore, in all the experiments described both brain and liver were minced at this concentration. In the experiments described above, there should have been no inhibition in brain cell UDS and 50% inhibition of liver cell UDS, if brain and liver cells responded to inhibition as did lung cells.

DISCUSSION

Low repair capacity in hamster brain cells

Repair synthesis in irradiated brain cells decreased by 70% as the hamster aged from 4–8 days to 39 days and remained at this low level until day 551 (Table 1, Fig. 2). The initial reduction in repair capacity resulted mainly from a drop in the proportion of

irradiated brain cells which exhibited UDS. Due to the high percentage of brain cells in S phase at days 4–8 (6.6%) compared with later ages (average of 0.4%) and to reports of DNA synthesis occurring in rats at early ages (Mori *et al.*, 1970; Altman, 1966), it is probable that during the 4–8-day period a significant proportion of the brain cells had still not entered the postmitotic stage. The decline in excision repair as brain cells apparently differentiate from the mitotic to the postmitotic state is in accord with the 10-fold decrease in methyl methanesulfonate- and N-acetoxy acetylaminofluorene-induced excision repair found as chick neural retina cells ceased replication (Karran *et al.*, 1977). U.v.-induced and chemically-induced DNA repair might be expected to respond similarly during the shift to the postmitotic state since recent evidence indicates a common enzyme or regulator for both types of repair in human fibroblasts (Brown *et al.*, 1979) as well as in yeast (Ruhland and Brendel, 1979).

The early decline in excision repair capacity of brain cells which we observed may reflect developmental regulation of DNA repair. If the decrease in DNA repair capacity in the brain allows accumulation of DNA lesions which interfere with transcription, cellular deterioration would ultimately result. Such deterioration would probably not be designed into the system but would more likely be a by-product of a phenotype which has a definite, positive selective value before or during the reproductive phase of life (Calow, 1978). The brain is the most energy consumptive organ of the body, as indicated by its large oxygen requirement. Although the human brain represents only 2% of the total body weight, it uses 20% of the total resting oxygen consumption (Iverson, 1979). The channeling of ATP to particular brain cell functions may require restriction of energy consumptive repair as well as replication.

Vol. 16 1981

Repair synthesis of the irradiated postmitotic brain cells was approx. 9.6% of that found in adult lung cells and 22.4% of that found in adult kidney cells (Gensler, 1981), suggesting that the postmitotic brain has a comparatively low ability to repair u.v.-irradiated DNA. Although the levels of UDS achieved were low, there was definite stimulation of unscheduled DNA synthesis by u.v. irradiation. In the present study, an incidence of 50 Jm^{-2} of u.v. radiation to brain cells isolated from adult hamsters resulted in a 9-fold stimulation of repair synthesis. This is in agreement with the work of Hübscher *et al.* (1979), who reported a 7- to 10-fold stimulation of [^{3}H] dTTP incorporation into DNA of young adult rat neuronal nuclei after exposure of the cells to u.v. radiation (40 Jm^{-2}). The similar increase in repair found in isolated nuclei supplemented with the riboside triphosphate form of thymine and in whole cells supplemented with thymidine suggests that the hamster brain cells had no permeability barrier to thymidine, had an adequate level of thymidine kinase to phosphorylate the thymidine exogenously added (3.9×10^{-7} mmoles/ml) and that intracellular thymidylate pools did not appreciably dilute the exogenously supplied thymidine. Ishikawa *et al.* (1978) found UDS in post-mitotic rat retinal ganglion cells irradiated with 60 Jm^{-2}, using NR-M_2 emulsion (Konishiroku Photo Co., Tokyo) and an exposure time of 4 weeks. Other tissues were not included in this study, so the relative excision repair capacity of these ganglion cells was not determined. Lieberman and Forbes (1973) found no u.v.-induced UDS in radioautographs of brain biopsies of New Zealand rabbits, even though vascular endothelial cells on the same slide did show UDS. However, they did not test for cell viability and brain cells are especially susceptible to oxygen deprivation.

The low repair capacity of the brain may result in vulnerability to aging lesions

The possible importance of the low level of DNA repair in the brain is that it could

lead to accumulation of DNA damage with age. Accumulation of single-stranded regions has been demonstrated in the DNA of brain, liver and heart cells of senescent mice (Chetsanga *et al.*, 1975; Chetsanga *et al.*, 1977; Price *et al.*, 1971; Murthy *et al.*, 1976). A correlation between u.v.-sensitivity of xeroderma pigmentosum (XP, deficient in excision repair) fibroblasts, as measured by loss of colony forming ability of cells from different patients, and age of onset of neurological abnormalities has been reported (Andrews *et al.*, 1978). If lack of repair of endogenous DNA lesions in XP individuals leads to the neurological abnormalities observed, it would imply that brain cells are particularly vulnerable to endogenous DNA damage, in accord with the low level of excision repair of u.v.-induced DNA lesions. One example of a spontaneous, repairable DNA lesion is depurination. This process is estimated to proceed at a rate of $k = 3 \times 10^{-11}$/s at 37°C and pH 7.4 (Lindahl and Nyberg, 1972). Several endonucleases from mammalian cells have been characterized as nicking DNA at apurinic sites, thus providing substrate for excision repair (Lindahl, 1979). Loss of neurons has been found in aged rats (Landfield *et al.*, 1977; Peng and Lee, 1979) and aging rhesus monkeys (Brizzee, 1975), so that neuronal loss may occur in normal animals and contribute to loss of function in neuronal tissue.

The existence of different levels of excision repair in different tissues implies that tissues with a low repair capacity can remove fewer lesions than tissues with high repair capacity. Thus, low repair capacity may result in relatively high vulnerability to DNA damaging agents. The following mechanism for loss of cell function with age in tissues with low repair capacities is proposed: (1) DNA damage occurs at a specific rate, (2) there is a net accumulation of DNA lesions if the rate of repair is lower than the rate at which damage occurs, (3) the unrepaired or partially repaired lesions lead to transcription termination (Hackett and Sauerbier, 1975; Nocentini, 1976), (4) premature transcription termination can result in decreased levels of proteins and (5) reduced protein levels lead to a loss of cellular and eventually tissue function.

Increased brain weight relative to body weight correlates well with increased maximum life span (Sacher, 1957) implying that an increase in the number of brain cells may be important in determining life span. We speculate that increased numbers of brain cells act as a reserve supply which becomes depleted with age due to the occurrence of endogenous DNA lesions and the low repair capacity of the post-mitotic brain. Thus, loss of neuronal cell functions due to accumulation of DNA lesions may be a signficant factor in aging.

Repair capacity of hamster liver cells

Our results show only 7% of liver cells performing u.v.-induced UDS throughout the age span studied, although this is probably a 2-fold underestimate due to the presence of an inhibitor in disrupted liver tissue (Fig. 4). Yager and Miller (1978) have reported u.v.-induced UDS in approx. 93% of primary cultured hepatocytes of another rodent, the rat. Differences in technique may explain some of the disparity in repair capacity between the animals and there may be a species variation as well. Yager and Miller selected for hepatocytes from the liver cells, used a different medium and a 6- to 12-fold longer labeling period than in the present study.

Margison *et al.* (1976) have shown that hamster liver has an extremely low capacity for *in vivo* excision of O^6-methylguanine, in contrast to a high level in rat liver (Margison and Kleihues, 1975). This indicates that repair capacity in the liver of rodents can be very

different and that the high level of repair of thymine dimers observed in rat liver is not inconsistent with the low level which we found in hamster livers.

SUMMARY

DNA repair was measured in brain and liver cells in terms of their ability to undergo unscheduled DNA synthesis (UDS) in response to u.v. radiation. The proportion of brain cells exhibiting u.v.-induced UDS decreased from 13.4 to 4.2% as hamsters aged from 4–8 days to 38–57 days and then remained at this low level at least to day 551, which is approx. ⅔ of the maximum life span of this strain of hamster. Repair synthesis in brain cells during this long period was approx. 9.6% of that found in adult lung cells, 22.4% of that found in adult kidney cells and, at most, 50% of that in adult liver cells. This suggests that the postmitotic brain has a low repair capacity compared to other tissues. If this rate of repair is less than the rate at which endogenous DNA damage occurs, then such damage would accumulate and perhaps contribute significantly to normal aging.

Acknowledgements—The helpful discussions with Drs. Harris and Carol Bernstein throughout this study were appreciated. This investigation was supported by grant 1-RO1-GM27219 from the National Institutes of Health and by a National Research Service award 5-T32-GM07239 from the National Institutes of Health.

Vol. 16 1981

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ANNOUNCEMENT

PRELIMINARY PROGRAM

SATELLITE SYMPOSIUM TO ISN MEETING: CELLULAR AND MOLECULAR MECHANISMS OF AGING IN THE NERVOUS SYSTEM

THIS symposium commemorates the pioneer work of GIUSEPPE LEVI, condensed in his comprehensive publications, *Physiopathology of Aging* (1933), and *Development and Senescence* (1946), constituting a major and early contribution of Italian neurobiology to the problem of aging of the nervous system. Changes in higher function of the nervous system during senescence have been related to loss of nerve cells, depleted synaptic population and deficits in neurotransmitter metabolism. In this symposium, cellular as well as molecular mechanisms of aging of the neuron will be discussed, mainly emphasizing: (a) the use of long-term cell and organ-cultures of neurons and glia, (b) changes in synaptic mechanisms including neurotransmitter biosynthesis and inactivation, membrane proteins and receptors, (c) diminished axoplasmic flow, and (d) changes in the general metabolism of the nerve cell, particularly glucose utilization.

Morphological work suggesting nerve cell loss, reduction in dendritic arborization and synaptic density will be critically evaluated in the light of recent biochemical findings in the same structures.

The design of new drugs and therapeutic attempts which might prevent, compensate for, or partially counteract the negative effect of aging on the nervous system as well as attempts to an "early diagnosis" of aging will be stressed.

Co-Chairmen: G. FILOGAMO, E. GIACOBINI, G. GIACOBINI and A. VERNADAKIS.

Local Organizing Committee: *Chairperson:* M-G. GIACOBINI-ROBECCHI (Turin).
Secretary: M. MARCHI (Genova).

Programme Committee: A. AMADUCCI (Italy), G. FILOGAMO (Italy), E. GIACOBINI (U.S.A.), A. VERNADAKIS (U.S.A.), G. GIACOBINI (Italy) and R. PAOLETTI (Italy).

Date: Monday, August 31–Wednesday, September 2, 1981 (2–3 days).
Invited Speakers: 20 (local expenses and registration free).
Travel Support: Being applied for.
Participants: 150–200 (registration fee—$50.00).
Place: Congress Centre, Hotel Billia, Saint Vincent, Aosta, Italy.
Location: Northern Italy, 40 miles from Turin Airport.
Activities: Invited lectures, discussions, posters and round tables.
The Proceedings of the Symposium will be published in a volume.
Deadline for receipt of abstracts (lectures and posters): 1 May, 1981.

FOR PREREGISTRATION FORMS WRITE TO:

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Vol.
16
1981

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OBITUARY

GEORGE A. SACHER

THE SUDDEN death of George Sacher has deprived gerontology of one of its most brilliant minds, and this Journal of an indefatigable Editor.

Sacher was the leading world authority on animal lifespan, on the longterm effects of radiation, and on the theory of genetic repair as a determinant of lifespan. He died shortly after returning from a visit to Canada, during which he presented a paper which was the summary of his life work: he was about to leave Argonne National Laboratories to become a Faculty member at the University of Chicago, his amla mater. Sacher joined the Manhattan Project in 1942 as Assistant Mathematician, then as Associate Biologist. In 1959 he became a Senior Biologist at Argonne. His publications cover a wide range of biometric subjects, including the relation of brain allometry to lifespan, the product of a laborious study of the data on mammalian longevities. Under his direction the requirements for longterm animal studies were defined and put into practice. George Sacher is survived by his wife Dorothea and his children, to whom we extend our deepest sympathy.

Vol.
16
1981

Dorothea Sacher writes of him:

> As George left home with the paramedics, still with book in hand, he ripped off his oxygen mask and said, "Tell everyone good-bye for me". With a smile and a wave of his hand, he was off in the ambulance.
>
> Much of his problem solving, theorizing, and rough manuscript composition was done on nature trails or in an isolated natural setting. His Argonne thinking place was the osage fence.
>
> The fence was planted by a farmer when the land was first settled many years before it became laboratory property. Our three children and I walked to the fence where three white deer were waiting to console us.
>
> I have been trying to decide just what George would have said to you as a good-bye. I am sure it would include urging you to persevere with utmost enthusiasm in your own field, to give credit to everyone assisting in your investigations thus encouraging younger scientists, to inspire students toward independent thought, to encourage them, too, to find a thinking place.

He will be kindly remembered, and deeply missed, by all who had the privilege of working with him.

ALEX COMFORT

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REPLICATION OF TOTAL AND MITOCHONDRIAL DNA IN RAT LIVER WITH AGING

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(*Received* 15 *October* 1980)

Vol.
16
1981

INTRODUCTION

SLOW-DOWN of cell growth and renewal, as well as decrease of energetic maintenance of cell functions are known to be linked with the decline in functional and adaptive capacities of an aging organism. Changes in nuclear (n-DNA) and mitochondrial (mt-DNA) liver DNA replication rates may underlie these events. DNA structure, polymerases activities as well as the rate of incorporation of labelled precursor into n-DNA and mt-DNA are decreased with aging (Vanjushin and Berdyshev, 1977; Gahan and Hurst, 1977). Besides, the increased quantity of circular dimers was discovered in population of mt-DNA in old mice tissues (Piko and Matsumoto, 1977).

However, the results of research on age-dependent changes of liver DNA replication using ^{3}H-thymidine are controversial. Samis *et al.* (1966) observed increased ^{3}H-thymidine incorporation into total DNA (t-DNA) of old rats without corresponding increase of mitotic index. The authors explained this discrepancy by the increase of DNA repair with aging. Bullock *et al.* (1978) found no changes in the intensity of ^{3}H-thymidine incorporation into rat liver t-DNA with aging. The increased n-DNA synthesis was observed in liver of 12- and 18-month old mice as compared to 9-month old animals, but there were no age-dependent changes in mt-DNA synthesis (Shima, 1975).

The purpose of present communication was to study t-DNA and mt-DNA replication in liver of adult and old rats.

MATERIALS AND METHODS

Adult (6 months, 170–250 g) and old (24 months, 300–400 g) female albino rats were used in this study. Animals were killed 10, 30, 70 and 120 min after administration of ^{3}H-thymidine (i.v. 11–19 Ci/mmole, 4μCi/g b.w., "Isotop", U.S.S.R.). Immediately after killing, pectoral and abdominal cavities were dissected and liver was perfused with 0.15 M NaCl (+2°C, 60–100 ml) through v. cava superior. Immediately after perfusion, the liver was removed and half of it was dipped in liquid nitrogen, while other half in STE-medium (+2°C, 0.3 M sucrose + 0.001 M EDTA + 0.005 M Tris–HCl, pH 7.4).

DNA was extracted from liver of animals kiiled in the morning in view of daily mitotic rhythm.

t-DNA from nitrogen-frozen liver was extracted by method of Habener *et al.* (1970) with some modifications (Obolenskaya and Levitsky, 1978). The tissue was homogenized in 5 vols of 6% sodium para-amino salicitate at +4°C.

Cell lysis was conducted in the medium containing 6% sodium para-amino salicilate, 2% SDS, 0.001 M EDTA for 30 min at 60°C. Lysate was extracted for 30 min at 25°C with a solution consisting of 88% phenol, 12% m-cresol, 0.1% 8-hydroxyquinoline. The extracts were chilled, twice centrifuged (4000 and 40,000 *g*), and the upper aqueous layer was exhaustively dialysed against 0.01 M-phosphate buffer (pH 6.8) to $A_{270} \leqslant 0.05$.

Purification of t-DNA from RNA and protein was conducted by hydroxyapatite column chromatography by method of Bernardi (1969). DNA in the peak fractions (5 ml) was precipitated with 5 ml of cold (+4°C) trichloroacetic acid (TCA) in the presence of added yeast RNA as coprecipitator (final concentration of nucleic acid did not exceed 120 μg/sample) on nitrocelliulose discs ("Synpor", CSSR, pore dia. = 1.5–2.5 μm). The composition of scintillator was: PPO—5 g, POPOP—100 mg, toluene—1 liter.

mt-DNA was extracted from the other part of the liver, which had been dipped in cold STE-medium. Tissue was passed through stainless steel press and homogenized in 8 vols of STE-medium with Porter–Elvhaime homogenizer. Homogenate was centrifuged at 800 *g* for 10 min. Supernatant was decanted and centrifuged at 10,000 *g* for 15 min. Mitochondrial pellet was washed three times under the same conditions. Three ml of 10% TCA was added to 0.5 ml of mitochondrial suspension (1 ml STE to 2 g liver) to remove acid-soluble compounds. Pellet was washed three times with 3 ml of 5% TCA. Phosphorus and lipids were removed with 96% ethanol (3 ml, 30 min), with mixture: ethanol–ether (3 : 1, v/v) (3 ml, 3 min, 100°C); with methanol–ether (1 : 1) (3 ml, 30 min) and 4 ml of ether. Pellet was dried at 37°C for 30 min. One ml of 1 M KOH was added to remove RNA and then the mixture was kept in thermostate at 37°C for 18 h. Then mixture was chilled at 0°C and 0.2 ml of cold 5 M $HClO_4$ was added. Pellet was washed twice with cold 1 M $HClO_4$ to remove rest ribonucleotides. DNA was extracted twice from the residue with 2 ml of 1 M $HClO_4$ at 80°C during 3 min and supernatants were united. The DNA content was determined spectrophotometrically by optical density difference at 268 and 284 nm (Tsanev and Markov, 1960).

Radioactivity was determined in 0.6 ml of supernatant placed in vials with 10 ml of dioxan scintillator.

Acid-soluble intracellular radioactivity was counted in perfused liver by precipitating acid-soluble fraction of sodium para-amino salicitate homogenate with 1 M $HClO_4$ at 3000 rev/min. 0.1 ml of supernatant was placed in scintillation vials together with 5 ml of dioxan scintillator.

The radioactivity was determined on a liquid scintillation spectrometer ("Intertechnique SL-30", France).

For statistical analysis the Wilcoxon–Mann–Whitney test was used.

The DNA specific radioactivity was expressed in cpm/mg DNA. The true DNA replication rate (relative radioactivity) was counted as:

$$\frac{\text{DNA specific radioactivity} \times \text{mg DNA per 1 g of liver}}{\text{acid-soluble radioactivity per 1 g of liver}}$$

and expressed in %.

Intracellular acid-soluble radioactivity was expressed in cpm/g of the liver.

RESULTS AND DISCUSSION

The kinetics of label incorporation in adult and old rat liver DNA is presented in Figs. 1 and 2. Regardless of the age, intensity of label incorporation was considerably higher in mt-DNA, than in t-DNA. The t-DNA specific radioactivity did not differ significantly in adult and old animals during all time periods. Similarly, there were no differences in the kinetics of mt-DNA specific radioactivity. There are two causes for

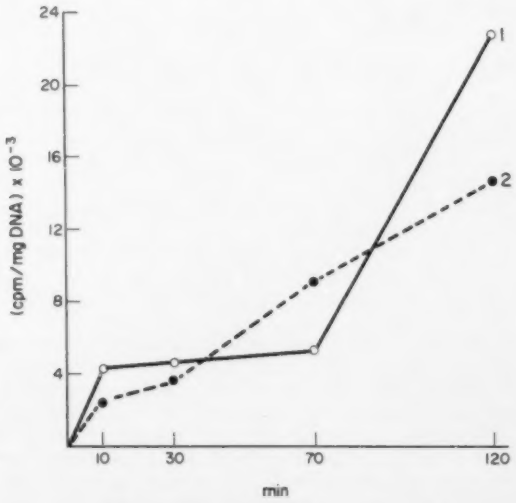

FIG. 1. Kinetics of t-DNA specific radioactivity in adult (1) and old (2) rat liver.

absence of age-dependent changes in the kinetics of t-DNA and mt-DNA specific radioactivities. The first are the equal DNA synthesis rates and labelled precursors concentrations in adult and old rat liver. The second is the higher DNA synthesis rate in combination with lower labelled precursors concentration in any age group. Thus, to determine the true DNA synthesis rate, it was necessary to estimate the tissue's acid-soluble radioactivity as an index of intracellular labelled precursor concentration.

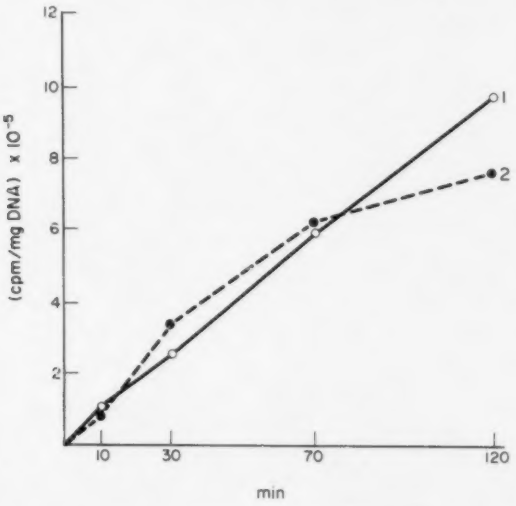

FIG. 2. Kinetics of mt-DNA specific radioactivity in adult (1) and old (2) rat liver.

Figure 3 illustrates the higher levels of intracellular acid-soluble radioactivity in the old liver for all studied periods. These age-dependent changes may result from higher permeability of old liver cells for thymidine (Ungemach and Hegner, 1979), decreased unlabelled precursors pool of old liver decreased aqueous liver phase/circulating blood volume ratio. Decrease of circulating blood volume/body mass ratio may be excluded in view of lack of age-dependent changes in label concentration in adult and old rat blood serum, 30, 70 and 120 min after i.v. administration of ^{3}H-thymidine (Levitsky, 1980).

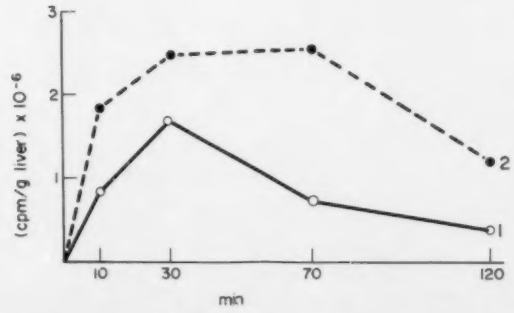

FIG. 3. Kinetics of intracellular acid-soluble radioactivity in adult (1) and old (2) rat liver.

Figures 4 and 5 demonstrate age-dependent peculiarities of the DNA relative radioactivity in the liver, being an index of true DNA synthesis rate since it shows the amount of intracellular DNA-incorporated radioactivity and does not depend on absolute concentration of labelled precursors. As is seen in Fig. 4, the t-DNA relative radioactivity of adult rat liver was significantly higher than that in old one at the 10th ($p < 0.01$), 70th ($p < 0.01$) and 120th ($p < 0.05$) minute. Such regularity was true for the mt-DNA relative radioactivity ($p < 0.001$) for all studied periods.

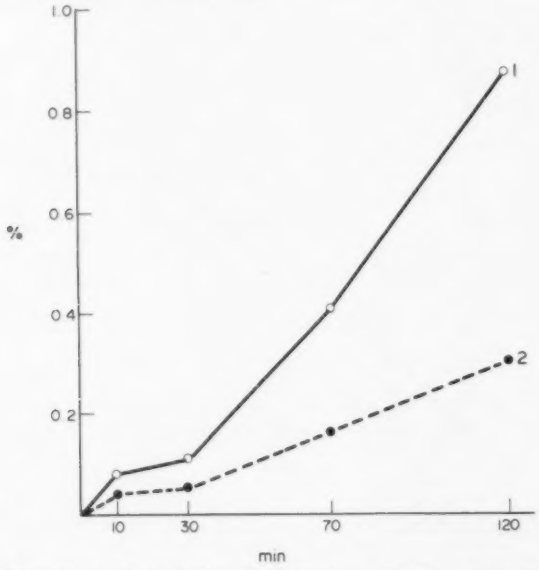

FIG. 4. Kinetics of t-DNA relative radioactivity in adult (1) and old (2) rat liver.

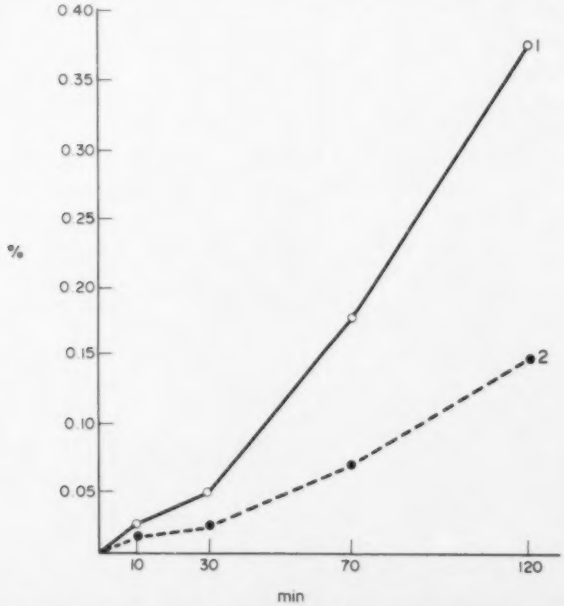

FIG. 5. Kinetics of mt-DNA relative radioactivity in adult (1) and old (2) rat liver.

Thus, there is a slow-down of the t-DNA and mt-DNA synthesis rates with aging.

It is unclear, however, whether the DNA synthesis is the synthesis of mt-DNA only in view of data on absence of the DNA replicative synthesis in liver as a tissue with low mitotic activity (Samis *et al.*, 1966). Our results on the DNA relative radioactivity showed a significant part of label being incorporated in t-DNA as compared with mt-DNA regardless of the time of labelling and animal age (Figs. 4 and 5).

Data on t-DNA and mt-DNA radioactivity per 1 g of liver may serve as clear indication for predominant label incorporation into t-DNA, as compared with mt-DNA. The data in Table 1 show that the mt-DNA radioactivity equals about ⅓ of the t-DNA radioactivity for all studied labelling periods. These differences were statistically significant for adult and old animals. Thus, the difference between the t-DNA and mt-DNA radioactivities estimated in 1 g of liver was the n-DNA radioactivity. In this connection, a note should be made of data by Tretjak *et al.* (1976) on major amount of label incorporated in liver n-DNA as a result of replicative synthesis.

TABLE 1. AGE-RELATED DIFFERENCES IN t-DNA AND mt-DNA RADIOACTIVITIES IN 1 g OF RAT LIVER DURING DIFFERENT LABELLING PERIODS (cpm/g tissue)

Animals' age (months)	DNA classes	Labelling periods (min) 10	30	70	120
6 months (adult)	t-DNA	919 ± 257	831 ± 195	2158 ± 278	3985 ± 754
	mt-DNA	134 ± 12	352 ± 51	736 ± 108	1182 ± 183
		$p < 0.01$	$p < 0.05$	$p < 0.001$	$p < 0.001$
24 months (old)	t-DNA	333 ± 72	696 ± 122	2225 ± 477	4728 ± 948
	mt-DNA	96 ± 8	358 ± 39	813 ± 18	784 ± 113
		$p < 0.01$	$p < 0.01$	$p < 0.01$	$p < 0.001$

Thus, the data obtained suggest a slow-down of the t-DNA and mt-DNA replication rates with aging.

The assumption of reciprocal control of nuclear and mitochondrial genomes (Baratz and Kuntzel, 1972) does not resolve the question about their effect on age-dependent changes of DNA replication. In any case, the data obtained are in agreement with the decrease of adaptive capacities of an aging organism (Frolkis, 1970). This age-dependent decrease of the DNA replication rate may result in insufficient cell energetic maintenance which may provoke a slowing of the renewal of cell populations. The latter is of interest in view of an important role played by the DNA replication in this renewal. In its turn, slowing of cell renewal may cause a decrease in liver energetic potential with aging.

SUMMARY

Replication of total (t-DNA) and mitochondrial (mt-DNA) liver DNA of adult (6 months) and old (24 months) female rats labelled with i.v. ^{3}H-thymidine has been studied. The most part of t-DNA radioactivity was shown to result from the replication of nuclear DNA (n-DNA). 10, 30, 70 and 120 min after labelling, intracellular acid-soluble ^{3}H-radioactivity of old rats was significantly higher than that of adult ones. This may be due to the change in precursors concentration with aging. Thus, despite the lack of differences between the specific radioactivities of t-DNA and mt-DNA of adult and old animals during all labelling periods used the true replication rate, i.e. specific DNA radioactivity/volume of intracellular pool of label (relative DNA radioactivity), was significantly higher in adult animals during all labelling periods.

Vol. 16 1981

Decrease of n-DNA and mt-DNA replication rate may lead to lowering of energetic potential and cell renewal in the liver with aging.

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Vol
16
198

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EARLY APPEARANCE AND RATE OF LIPOFUSCIN PIGMENT ACCUMULATION IN HUMAN MYOCARDIUM

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Vol.
16
1981

INTRODUCTION

LIPOFUSCIN pigment accumulation in the human heart has been observed to increase with age (Strehler *et al.*, 1959; McMillan and Lev, 1962; Andrezej and Buczynski, 1972). McMillan and Lev (1962) reported an absence of pigment in the myocardium of persons below 10 yr of age. With the advancement of age, lipofuscin pigment has been observed to increase significantly in the ventricle of rat (Reichel, 1968) and dog (Munnell and Getty, 1968). There is no significant report available on the rate of lipofuscin pigment accumulation in human myocardium except that of Strehler *et al.* (1959).

The present study has been carried out with a view: (a) to determine the earliest onset of lipofuscin pigment and (b) to establish qualitative and quantitative accumulation of lipofuscin pigment in the left ventricular myocardium.

MATERIALS AND METHODS

The human hearts (left ventricular myocardium) of 67 autopsy cases from 1 to 70 yr of age were used in the present study. The material from accidental cases was collected from N.R.S. Medical College, Calcutta, India. Every care was taken to get the material from healthy normal persons within 24 h after death. The tissue was fixed in 10% neutral buffered formalin immediately after autopsy. Paraffin sections were cut at 4 μ and stained with Sudan black B and alcian blue/periodic acid schiff for lipofuscins.

Autofluorescence technique was employed for visualizing lipofuscin pigments. Paraffin sections were mounted, after deparaffinisation, in high purity non-fluorescence paraffin oil and examined under ultraviolet light in the dark field of the microscope (Leitz Ortholux). The light source consisted of an Osram HBO- 200 W mercury arc lamp which emits light at more than 400 μ wavelength. The ultraviolet light was passed through a blue filter BG-12, a dark field condenser (D 1.2-1.4), non-fluorescence oil, the slide sections mounted in non-fluorescence oil, objective (0.65), a barrier filter (K 530) and then an eye piece.

Rate of lipofuscin pigment accumulation in the human myocardium was measured by the same method as was proposed by Chalkley (1943) and modified by Strehler *et al.* (1959). The grid containing 100 squares and consisting of 121 intersections was placed in the ocular and then superimposed on the selected fields of the myocardium. If anyone of these intersections was found to be superimposed over the fluorescing pigment granules in the field, it was considered as a hit. The total number of hits per field was counted and repeated for ten fields. The average was ten taken. The total number of hits was divided by the total number of possible hits and expressed as a percentage.

OBSERVATIONS

Histopathological and autofluorescence studies were made to examine the accumulation of lipofuscin pigment in the left ventricular myocardium. The present qualitative and quantitative observations in different age groups are given below.

Qualitative observations

Lipofuscin pigment granules were first observed to appear at 9 yr of age near the poles of nuclei of myocardial muscle cells. These pigment granules were located at some places

with fluorescence light as well as with staining reactions. No pigment granules were, however, observed in persons below 9 yr of age. The qualitative studies revealed progressive increase of lipofuscin pigment accumulation with age in the myocardium. A few small lipofuscin pigment granules, scattered at places in the myocardium, were observed at 9 yr of age. In the second decade, the number of pigment granules had increased. They continued to increase in number and started accumulating in the third decade. During the fourth decade the pigment granules were observed quite close to each other and in the fifth decade, the pigment bodies appeared in accumulated and concentrated masses. Similarly, in the sixth and the seventh decades, more accumulated masses of the pigments were observed and pigment bodies appeared to be larger in size.

Quantitative observations

Mean values of the quantitative increase of pigment accumulation with age in the ventricular myocardium are shown in Table 1. A progressive increase of lipofuscin pigment accumulation was observed in the ventricular myocardium with advancing age (Table 1, Fig. 1). The differences in pigment accumulation between the second and third decades onwards were significant (< 0.001). No pigment was, however, quantified in the first decade as the onset of pigment started at 9 yr of age. The pigment was found to increase statistically at a rate of 0.609% pigment vol./myocardial vol./decade. Hence, the increase in per cent pigment by vol./per cent myocardium by vol./year was 0.0609.

TABLE 1. ACCUMULATION OF LIPOFUSCIN PIGMENT IN THE VENTRICULAR MYOCARDIUM IN RELATION TO AGE

Age group	Age (yr)	No. of subjects	% pigment by vol./ % myocardium by vol.
A	11–20	8	0.47 ± 0.0399
B	21–30	11	1.07 ± 0.0977
C	31–40	12	1.69 ± 0.1220
D	41–50	10	2.35 ± 0.1460
E	51–60	10	2.87 ± 0.1776
F	61–70	16	3.65 ± 0.1678

This table does not include persons below 11 yr of age since the onset of pigment started at 9 yr of age.

$p < 0.001$ = A and B, A and C, A and D, A and E, A and F, B and C, B and D, B and E, B and F, C and E, C and F, D and F. $p < 0.01$ = C and D, E and F, $p < 0.05$ = D and E.

DISCUSSION

Hamperl (1934), Strehler *et al.* (1959) and McMillan and Lev (1962) reported the absence of lipofuscin pigments below 10 yr of age in human ventricular myocardium. Strehler *et al.* (1959) demonstrated absence of lipofuscin pigments in very young individuals but they were always present in old individuals. The present study revealed the earliest appearance of lipofuscin pigments at 9 yr of age in the left ventricular myocardium.

In the present investigation, the pigment bodies in human beings were found at the poles of myocardial nuclei. Similar observations were also made by Hamperl (1934) and

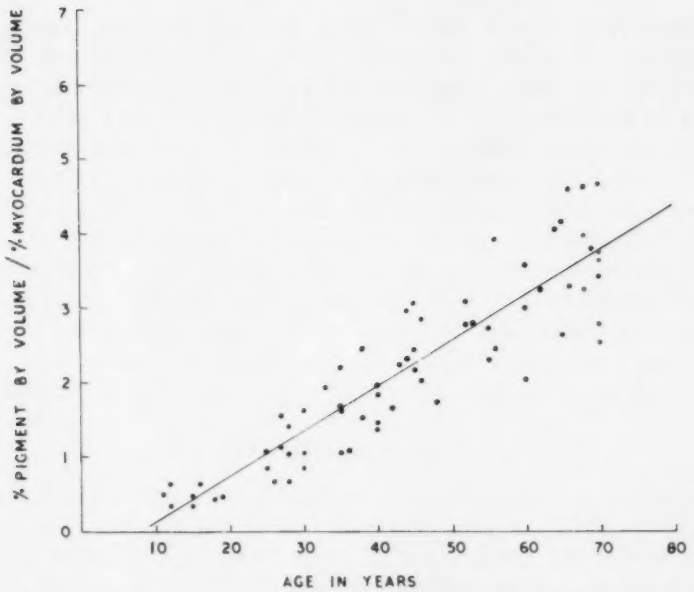

FIG. 1. Per cent pigment by vol./ per cent myocardium by vol. vs age for human beings older than 11 yr of age. Linear regression data: $N = 67$; $\overline{X}$ (mean age) = 44.1045 yr; $\overline{y} = 2.1924\%$ pigment by vol./percent myocardium by vol.; $\zeta = 0.9069$, $p < 0.05$; $byx = 0.0609\%$ pigment by vol./percent myocardium vol./year; $y = -0.4899 + 0.0609x$.

Strehler *et al.* (1959). The pigments were observed in concentrated masses in old individuals. Munnell and Getty (1968) also revealed the clumping of lipofuscin granules in dog with the advancement of age.

The present qualitative and quantitative observations revealed progressive accumulation of lipofuscin pigments in human ventricular myocardium with advancing age (Table 1, Fig. 1).

Lipofuscin pigment increased at a linear rate of 0.67% pigment vol./myocardial vol./ decade in human ventricular myocardium (Strehler *et al.*, 1959). So it would be equal to 0.067% pigment vol./myocardial vol./year. The present investigation revealed that lipofuscin pigment increased at a linear rate of 0.0609% pigment vol./myocardial vol./year.

Munnell and Getty (1968) revealed the accumulation of lipofuscin pigment at a linear rate of 0.36% pigment vol./myocardial vol./year in the ventricular myocardium of dog. They discussed the correlation of pigment accumulation between man and dog. Munnell and Getty (1968) stated, from their observations in dog and from the observations of Strehler *et al.* (1959) in man, that the pigment accumulated in dog approx. 5.5 times faster than in man. This could be expected since man lives approx. 5 times longer than dog.

Strehler *et al.* (1959) postulated that pigment accumulation must have some definite effect on cardiac muscles. They proposed three alternative possibilites: (1) the pigment may interfere with the efficient function of heart muscle; (2) the inclusions may represent some advantageous functional unit developed to compensate for some other loss and (3) these bodies may not function appreciably either positively or negatively.

Strehler *et al.* (1959) and Travis (1972) considered that the accumulation of lipofuscin pigment may affect the efficient working of the heart muscle with age. Samorajski *et al.*

Vol. 16 1981

(1968) and Goyal (1978) were of the opinion that the pigment accumulation may affect all sorts of neuronal physiology and possibly result in a loss of cells. Munnell and Getty (1968) thought that the pigment might have some advantageous functional unit developed to compensate for some other loss. They believed it to be due to the position of the pigment at the poles of myocardial nuclei, where the pigment masses could exert little mechanical effect either on contractile mechanisms or on the diffusion of substances throughout the sarcoplasm. Björkerud (1963) and Björkerud and Cummins (1963) found considerable oxygen uptake in lipofuscin of bovine cardiac muscle. Lipofuscins were found to contain hemoproteins and carotenoids (Björkerud, 1964). Karnaukhov (1973) and Karnaukhov *et al.* (1973) demonstrated that lipofuscin pigments contain carotenoids, myoglobin and some other respiratory enzymes. They suggested that lipofuscin functions in forming the intracellular stock energy and providing energy requirements under hypoxic conditions. Further studies will be performed to observe the physiology of lipofuscin pigments.

SUMMARY

Lipofuscin pigment granules appeared at 9 yr of age in the left ventricular myocardium. The pigment was found to increase progressively with age and to congregate in masses in the myocardium of old individuals. The pigment has been observed to increase at a rate of 0.0609% pigment vol./myocardial vol./year.

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EFFECT OF TEMPERATURE AND DIFFERENT SEX RATIOS ON PHYSICAL ACTIVITY AND LIFE SPAN IN THE ADULT HOUSEFLY, *MUSCA DOMESTICA*

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Vol. 16 1981

INTRODUCTION

IT IS generally believed that the maximum life span of organisms is a genetically determined phenomenon, but the achieved longevity is strongly influenced by certain environmental conditions (for review see Clark, 1964; Lints, 1978). The life spans of insects, like the other poikilotherms are, in general, inversely proportional to the ambient temperature (see reviews by Rockstein and Miquel, 1973; Sohal, 1976). For example, the average life span of *Drosophila melanogaster* was reported to be 120 days at 10°C, but only 13.6 days at 30°C (Loeb and Northrop, 1917). The mechanism by which elevation in temperature within the viable range of the species shortens longevity is still obscure, although it has been suggested that higher temperature accelerates the rate of aging by stimulating the metabolic rate (Miquel *et al.*, 1976). Besides affecting the basal metabolic rate, temperature also has a direct effect on the level of physical activity in insects (Pearl, 1928). The effects of temperature on the levels of physical activity have not been well investigated in the flying insects due to the lack of adequate techniques.

Another factor which greatly affects the intensity of physical activity and the consequent metabolic expenditure involves mating activity. The sex ratios within a population have a significant effect on the mating behavior of many insects such as the housefly. Previous studies in this laboratory have indicated that sex ratios of the housefly also have a strong effect on longevity (Ragland and Sohal, 1973, 1975). The physiological basis of this relationship is presently rather obscure.

The objective of the present study was to explore the nature of the relationship between physical activity and life span in the houseflies. The levels of physical activity were altered experimentally by variations in the ambient temperature and sexual composition of the populations. The flying and walking activities of the flies were measured by a new technique, employing a radar-Doppler system, which was developed in this laboratory (Buchan and Sattelle, 1979).

MATERIALS AND METHODS

Rearing the flies

Eggs obtained from 7 to 10-day-old adult houseflies, belonging to a stock maintained at the Zoology Department, University of Cambridge, were placed in grass-meal larval medium. After eclosion, the flies were kept in 3-l clear plastic jars at 28°C, under 24 h lighted environment, and fed on sucrose and water.

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Life spans

The effects of temperature on life span were measured at 18 and 28°C in groups of 150 flies consisting of equal numbers of males and females. The number of dead flies was counted every 1–2 days. In order to elucidate the effects of sex ratios on mortality, the life spans of three different populations composed of 50 male, 50 female, and 40 male and 10 female flies were also computed.

Sex ratios. Certain aspects of the sexual behavior of the houseflies, reported previously from this laboratory (Ragland and Sohal, 1973), were employed for altering the mating activity of the flies, and are briefly described here in order to facilitate the understanding of the experimental design of this study. Female houseflies are monogamous, i.e. they mate usually once in life, whereafter they resist copulatory attempts of the males. The males, on the other hand, attempt to mate numerous times. Quite obviously, in populations with a high male to female ratio, all the females are mated quite quickly which renders them resistant to further copulation. This would tend to increase the flight activity of the males for sexually receptive females. Levels of physical activity were compared in three different populations of houseflies with the following compositions: group A, 50 males; group B, 50 females; group C, 40 males and 10 females. Mating-related activity in group C males would be expected to be relatively high. As mentioned above survival records of the flies in each population were also obtained.

Measurement of physical activity

The effects of different sex ratios and temperature on the walking and flying activities of houseflies were measured by using a radar-Doppler device developed in this laboratory (Buchan and Satelle, 1979).

Radar-Doppler is non-intrusive and the insect under observation is subjected to only 0.001–0.02 mW cm^{-6}. Power densities of 10 mW cm^{-2} and above are known to perturb insect activity (Riley, 1974 reference to unpublished work of Tanner A. J., 1971). Flying and walking activity was measured simultaneously by passing the Doppler signal through two filters and subsequently into two channels (Buchan, in preparation). Flies used as radar targets reflect sufficient energy to give usable Doppler-shifted signals within two frequency ranges, (a) up to 5 Hz (walking) and (b) from 5 to 10 Hz (flying). Output from two channels is displayed as either a bar chart, an integral or a totalized count, all with re-setting facility for periods of time, generally 1 h.

Plastic containers, housing the flies, were placed in a marked central position, one at a time, in a metal environmental cabinet fitted with a light source. Through one of the walls of the cabinet the radar module, containing both receiver and transmitter, was fixed. On closing the cabinet door, the radiated electromagnetic energy is restricted to the confines of the cabinet and a standing wave pattern is set up. Disturbance of the pattern caused by the movement of the flies gives rise to a Doppler-shifted signal at the input to the receiver; electronic signal processing arranges the output displays as mentioned above.

Measuring activity against temperature was a semi-automatic experiment. An ice pail constructed of expanded polystyrene was chosen to house the flies as this material is transluscent to light. The thermal properties of the pail ensured that temperature would change slowly over a period of hours. A hole was cut in the lid of the pail and a tin can inserted 2/3 of its depth into the hole (Fig. 1c). Tests showed that the internal temperature of the pail would fall by 10–15°C when −10°C crushed ice was placed in the can.

For the experiment a random selection of 11 female and 7 male flies was made. The flies were placed in the ice pail with ample sugar and water. With the pail in the environmental cabinet, they were allowed to settle down for a 24-h period. After this period, when it was clear that the activity had remained steady for a period of approx. 2 h, the −10°C crushed ice was placed in the can. Temperature in the pail fell to 15°C within 15 min (Fig. 1b). Activity and temperature of the flies were monitored for a further 24 h, the temperature in the pail obviously changing during that period.

Student's *t* test was used for determination of the significance of the differences in life spans and physical activity between the different groups.

RESULTS

Life spans

The survivorship data of the housefly populations maintained at 18 and 28°C are provided in Table 1. The average life span of both the male and female flies was significantly longer at 18°C ($p < 0.001$) than at 28°C.

The effects of different sex ratios on life span, presented in Table 1 show that the average life span of female flies in group B, which was composed of females only, was significantly longer ($p < 0.01$) than that of the males, either isolated (group A) or maintained with a few females (group C). The average life span of female flies in group C, which had a ratio of 4 male: 1 female, was, however, significantly shorter than the isolated females in group B. The average life span of male flies in the mixed population (group C) was significantly shorter than that of the males in population A ($p < 0.05$).

TABLE 1. EFFECTS OF SEX RATIOS AND AMBIENT TEMPERATURE ON LIFE SPAN OF ADULT HOUSEFLIES

Group	Temperature (°C)	No. of males	No. of females	Average life span (days)
A	28	50	—	25.4 ± 1.6
B	28	—	50	30.5 ± 2.8
C	28	40	10	M 19.8 ± 1.6
				F 19.3 ± 2.3
—	18	75	75	M 37.5 ± 2.1
				F 42.7 ± 2.2
—	28	75	75	M 21.8 ± 1.2
				F 24.5 ± 1.5

Results are ± SEM.
M, males; F, females.

Physical activity

Preliminary studies had indicated that houseflies kept under a constant light environment did not exhibit any detectable circadian rhythmicity in the level of their physical activity, therefore it was assumed that valid comparisons could be made between the data obtained in various groups at different times of the day. The highest levels of walking and flying activities in the houseflies were observed around 4–5 days of age: consequently, flies of this age were used for measuring the effects of various experimental conditions on movement.

Temperature. The effects of temperature on walking and flying activities of the male flies are presented in Fig. 2. Houseflies used in this experiment did not exhibit any detectable walking or flying movement at 15°C but commenced walking at 15.5°C (Fig. 1).

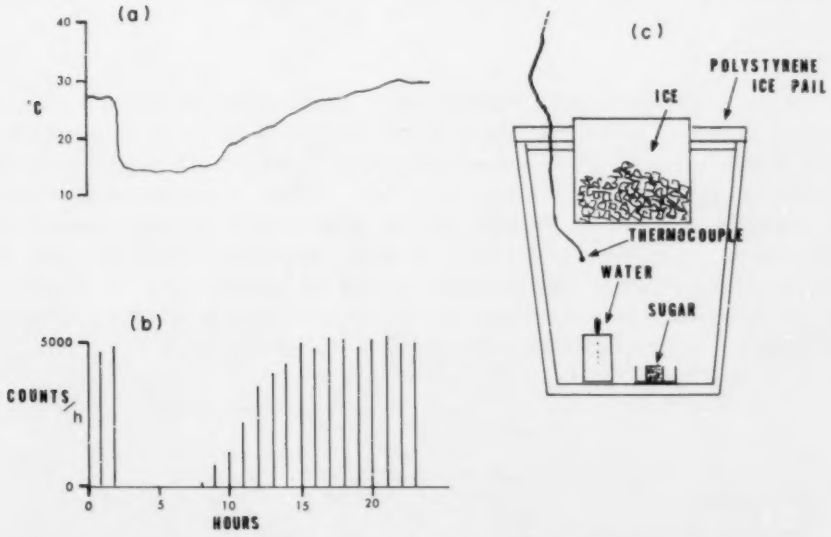

FIG. 1. (a) A chart recording showing temperature inside ice pail during the experiment. Temperature was measured by thermocouple probe. (b) record of flying activity throughout experimental period. Walking activity followed a similar pattern but is not included in the figure. (c) Section through the 3-l ice pail showing the 0.5-l tin, inserted in the pail lid. Flies were free to move throughout the free volume of the pail.

Vol. 16 1981

There was an approx. five-fold increase in the walking activity between 15.5 and 17.5°C and a further tripling of walking between 17.5 and 24.5°C. The flying movement, however, was first detectable around 17.5°C and subsequently increased about 10 times, reaching a peak at 24.5°C. Thereafter, both the walking and the flying activities remained at a plateau level up to 26°C and showed a slight decline between 26 and 28°C. Thus, the steepest effects of temperature on activity were felt within a narrow range which is different for walking and flying. For example, there was a three-fold increase in walking between 17 and 18°C, whereas the greatest stimulation for flying occurred between 20.5 and 21.5°C.

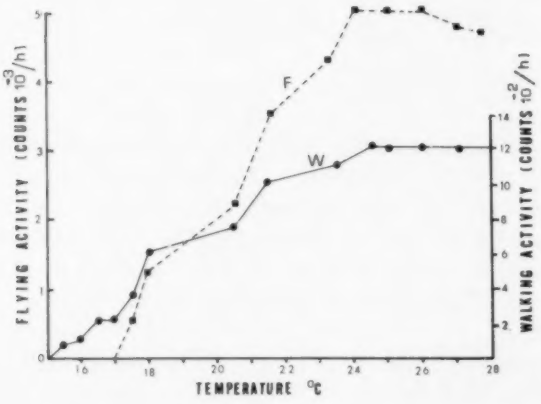

FIG. 2. Graphic representation of the effect of temperature on walking (W) and flying (F) activity of adult houseflies.

Variations in sex ratios. The effects of different sex ratios on the walking and the flying activities in the housefly populations are presented in Table 2. Population B, composed of 50 females, exhibited a significantly lower level of walking activity ($p < 0.05$) than populations A and C. There were no significant differences in total walking activity between populations A and C, whereas considerable differences were observed in the total flying activity of the various populations. Again, the lowest level of flying was noted in population B consisting of females only, whereas the highest flying activity occurred in group C, composed of 80% males and 20% females. According to the flight frequency, different populations were ranked as follows: C > A > B ($p < 0.05$).

TABLE 2. PHYSICAL ACTIVITY IN HOUSEFLY POPULATIONS WITH DIFFERENT SEX RATIOS

Population	Composition	Walking	Flying	N
A	50 M	2400 ± 120	15,360 ± 460	12
B	50 F	2100 ± 103	13,760 ± 410	15
C	40 M + 10 F	2630 ± 130	21,364 ± 1050	7

Values are ± S.D.
M, males; F, females.

DISCUSSION

The results of the present study indicate that the life span of adult houseflies is strongly influenced by the environmental temperature and sexual composition of the populations. Both the walking and flying activities are tremendously stimulated by temperature, with the former exhibiting a 15-fold, and the latter a 10-fold increase within a range of less than 10°C. The life span, on the other hand, is considerably shortened at higher temperatures. Similarly, in populations with different sex ratios, the levels of physical activity are, in general, inversely related to the life span.

Although the life-shortening effects of higher ambient temperatures on poikilotherms are well known, a physiological explanation of this relationship has remained controversial. According to one view, originally advanced by Pearl (1928) and generally referred to as the "rate of living theory", the shorter life spans at higher temperature are due to an increase in the metabolic rate, which brings about an acceleration in the rate of aging. An opposing view, advocated by Maynard Smith (1963), claims that shorter longevity at higher temperatures is not due to a faster rate of senescence, but to the "vitality threshold" needed for survival, which increases with temperature. Subsequent studies, testing the validity of these conflicting explanations, which were mostly conducted on *Drosophila,* have not clearly supported the "threshold" or the "rate of living" hypothesis (for review see Lints, 1971; Sohal, 1976). It was pointed out by Sohal (1976) that apparently a major cause of the conflict was the erroneous assumption by some investigators that the ambient temperature was linearly proportional to metabolic rate. In fact, temperature-induced increase in the rate of oxygen consumption of poikilotherms is due to two distinct contributory factors: (a) basal metabolic rate and (b) physical activity (Prosser, 1973). Whereas the basal metabolic rate may increase uniformly with temperature, the results of the present study indicate that the effects of temperature on walking and flying activities of the adult housefly are certainly not uniformly linear. Flying exerts extremely high metabolic demands in insects, for example, in some dipterans oxygen consumption has been reported to increase about 100 times during flying as compared to the sitting state (Davis and Fraenkel, 1940). Thus, the temperature effects on physical activity must be taken into account in evaluating the relationship between metabolic rate and life span. As shown in this study, there is considerable variation in insect movement at different temperatures. It would seem that the validity of the above mentioned alternate explanations concerning the relationship between temperature and life span cannot be adequately tested without accompanying measurements of oxygen consumption.

The effects of varied sex ratios on life span and activity, as revealed in the present study, have several noteworthy features. For instances, in populations consisting of males (population A) and females (population B) only, the average life span of the male flies is shorter than that of the female flies. Similar differences in the longevity between males and females exist in many animal species. The results of this study indicate that the males exhibit greater physical activity than females and thus probably consume relatively greater amounts of oxygen. In a previous study where the flight activity of the two sexes was abolished by confining flies individually in small vials, the life spans of the male and female houseflies became equal to each other (Ragland and Sohal, 1973). It is therefore possible that life span differences between the two sexes in the housefly may be related to the underlying differences in physical activity and metabolic rate. Individual variations in the life spans of male flies kept under identical conditions have been related to differences in levels of physical activity (Sohal and Buchan, 1981).

Vol. 16 1981

The average life spans of both the males and the females in population C, with a 4:1 male to female ratio, are significantly shorter ($p < 0.05$) than the separate males and females in populations A and B. The level of physical activity in population C is also greater than in populations A and B, which again indicates an inverse relationship between the level of physical activity and life span in the houseflies.

SUMMARY

The objective of this investigation was to further examine the relationship between physical activity and life span in the adult housefly. Levels of physical activity were experimentally varied by alterations in the ambient temperature and sex ratios of the populations. In agreement with previous studies, life span of the flies was significantly longer at 18°C than at 28°C. Female flies lived longer than males. Male flies in a population with a high male to female ratio lived a shorter period than their counterparts without any females. These variations in life span seem to be related to differences in the level of physical activity of the flies. The walking and flying activities of the various groups were measured separately by a radar-Doppler system. Walking activity increases about 15-fold, whereas flying activity increased more than 10 times between 15 and 24.5°C; thereafter there was a slight decline in both up to 28°C.

Populations with female flies only exhibited lower levels of physical activity as compared to populations of males only. Populations with a high male to female ratio had the highest levels of walking and flying activities. The life spans of the flies were thus inversely related to the level of physical activity. Results of this study support the concept that the rate of metabolic expenditure has a strong influence on life span. The nature of the underlying mechanism is, however, as yet obscure.

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ALTERATIONS IN TOTAL CONTENT AND SOLUBILITY CHARACTERISTICS OF PROTEINS IN RAT BRAIN AND LIVER DURING AGEING AND CENTROPHENOXINE TREATMENT*

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Vol. 16 1981

INTRODUCTION

Verzár and his school started the experimental studies of collagen heat-denaturation in 1955. They showed in a series of experiments that the aged tail tendons of rats were solubilized by thermic treatment at 64°C to a much lower extent, and at the same time, the old, denatured tendons possessed a much higher contraction force than the young ones (Verzár, 1955*a*, 1955*b*, 1956, 1964, 1969; Verzár and Freydberg-Lucas, 1958; Verzár *et al.*, 1965, 1972; Verzár and Zs.-Nagy, 1970, etc.). It has also been observed that there is a progressive decrease in the acid-solubility of skin collagen with age (Sinex, 1968). These experimental findings were explained by supposing an age-dependent increase of cross-linking of collagen. Although a great mass of data has been obtained during the last 10 years regarding the biochemical character of the cross-links present in the collagen fibers (see Bailey *et al.*, 1974; Balázs, 1977), the mechanism of the age-related cross-link formation is still an open question. It is quite clear that the reducible cross-links of aldimine type present in large amounts in the young collagen disappear during maturation, nevertheless, there are contradictory data regarding the possibility of *in vivo* reduction of these bonds to covalent cross-links (Bailey and Peach, 1971; Mechanic *et al.*, 1971; Deshmukh and Nimni, 1972; Robins *et al.*, 1973). Bailey *et al.* (1974) are of the opinion that the stable cross-links present in the insoluble old collagen may derive most probably from the reducible cross-links through a spontaneous conversion. This assumption is shared also by Balázs (1977).

The fact that we do not know exactly the mechanisms of age-related cross-link formation cannot decrease the biological significance of these post-translational changes of proteins. It seems to be possible that similar alterations may take place in all proteins of living systems. As a matter of fact, the method of thermal denaturation has revealed interesting age-related changes in the behaviour of DNA in isolated chromatin (Hahn and Verzár, 1963; Hahn, 1963, 1964; Phytilä and Sherman, 1968; Hahn and Fritz, 1966; Kurtz and Sinex, 1967; Verzár *et al.*, 1972). These alterations proved to be dependent on the presence of proteins (Hahn, 1963; Russel *et al.*, 1966).

On the basis of the above data one can suppose that the post-translational modification of the protein structure may be a general biological phenomenon. An age-dependent increase of cross-linking of tissue proteins may influence some basic characteristics of the proteins, e.g. the water-solubility of them. Unfortunately, however, the water-solubility

*Dedicated to the memory of Prof. Fritz Verzár.

of proteins is a very confused term. Here we list some data taken from the literature to illustrate the situation.

Medvedeva (1937) considered as water-soluble the proteins found in the liquid obtained from animal tissues by using the hydraulic press of Buchner (1897). According to her findings, both the total and the water-insoluble fractions of proteins increased considerably in the liver and muscle tissues of rats between the ages of two months and two years. At the same time she described an increased total protein content in the brain of old rats, however, the brain proteins passed totally into the press-liquid as in the young and adult age groups. It is noteworthy that protein contents were determined in these experiments by measuring nitrogen concentrations with the classical Kjeldahl method (Medvedeva, 1937).

In more recent experiments the method of Lowry *et al.* (1951) was used for protein determinations. For instance, Sessa and Perez (1975) homogenized rat brains in 10 vol. 0.32 M sucrose by Potter homogenizer and then centrifuged at 1000 ***g*** for 10 min. The proteins remaining in the supernatant were considered as water-soluble amounting to about 77% of the total proteins. No old animals were involved in this study. Other authors (Grossfeld and Shooter, 1971) homogenized mouse brain in 0.32 M sucrose buffered with Tris to pH 7.6 and then centrifuged at 100,000 ***g*** for 2 h. They described about 35% of total proteins as water-soluble. In this work mice up to an age of 700 days were involved and no age-dependent changes were detected. Again others (Naber *et al.*, 1979) studied human brains obtained at autopsy 12–18 h after death. They homogenized under quite different conditions (0.038 M Tris–HCl, 0.025 M sucrose, pH 8.4) as compared to the methods cited above, and centrifuged at 250,000 ***g*** for 95 min. Under such circumstances, the water-soluble proteins remaining in the supernatant amounted to about 25–30% of the total proteins. There was an age-dependent increase in this percentage between 16 and 91 years of age, accompanied by a slight decrease in total proteins.

Apart from the differences in preparing water-soluble proteins, there is the further problem of interpretation of the results. Namely, the reported total protein contents of the brain tissues are quite different; they vary from 50 to 120 mg protein per g wet weight of the brain (Naber *et al.*, 1979; Grossfeld and Shooter, 1971; Sessa and Perez, 1975; Toews and Horrocks, 1976). There is no doubt that these differences are due to the incomplete "exploration" of the proteins for the Lowry procedure or to interferences of some compounds. A detailed study of this problem is given by Grossfeld and Shooter (1971). Suitable modifications of the original method of Lowry *et al.* (1951) can be used to overcome these difficulties (for details see Peterson, 1979).

Considering the data cited above, it seems to us quite necessary to define precisely the experimental conditions of preparation and measurement of water-soluble and total proteins, if we intend to follow eventual age-dependent alterations. The present paper describes the results obtained by using a standardized method on the liver and brain of young, adult and old rats. Furthermore, we present data on the effect of centrophenoxine (CPH) on the total protein as well as the water-soluble protein contents of these tissues. CPH was administered *in vivo* to old animals. As is known, this drug is able to reduce the lipofuscin content of the brain cells and myocardium. It improves a series of brain functions both in animals and humans (for details see Zs.-Nagy *et al.*, 1979).

MATERIAL AND METHODS

The experiments were carried out on CFY rats of our own breed. This Wistar-derived strain displays a medium life-span of about 2 years, and its maximum life expectancy is about 39–40 months. In our experiments the

term "young" indicates animals of 1–4 months, the "adults" were of 15 months, and the "old" rats of 25–30 months of age. The animals were kept under normal laboratory conditions during their life. They were killed by decapitation and the tissue samples were removed from the liver and the parietal brain cortex as quickly as possible (within 1 min). The samples were immediately frozen and kept at −20°C before homogenization.

Separation of protein fractions

Water-soluble and water-insoluble proteins (WSP and WIP, respectively) were separated using the method of Hassell and Hand (1974). This method involved a homogenization of tissue pieces in water (10 mg/ml) by means of a Potter-Elvehjem homogenizer. The conditions of homogenization were kept as constant as possible, i.e. ten strokes, ice-cooling, identical motor-speed. Usually a total volume of 10 ml was used. An aliquot of 1 ml of the homogenate was taken and centrifuged at 500 **g** for 10 min in a Janetzki T30 type instrument. The supernatant contained the WSP, whereas the sediment represented the WIP.

Electron microscopy

The sub-microscopic composition of the WSP and WIP was controlled as follows: the WIP pellet was fixed in 2.5% glutaraldehyde buffered with sodium cacodylate (0.2 M, pH 7.2) for 2 h at 4°C, then washed in the buffer for a night. Post-fixation was made in 1% OsO_4 for 1 h in the same buffer. Dehydration in ethanol, embedding in Spurr's resin (Spurr, 1969). The WSP was processed in the same way with the only difference that twice-concentrated fixative was added to the supernatant in 1:1 proportion and the fixed WSP was pelleted by centrifugation after 2 h. Sections were cut on LKB Ultrotome III, and contrasted by uranyl-acetate and lead citrate as usual. Micrographs were taken with a JEOL JEM 100B electron microscope.

Vol. 16 1981

Protein determinations

The WSP-containing supernatant was separated, then the WIP sediment was resuspended in 1 ml of water. One millilitre of 10% trichloroacetic acid (TCA) was added to each fraction, in order to precipitate the proteins. After 10 min we centrifuged again at 500 **g** for 10 min, and the sediments were resolubilized in 3 ml of 1 M NaOH at room temperature during a night.

Protein contents of the alkali solutions were determined using the method of Lowry *et al.* (1951) as modified by Markwell *et al.* (1978). Essential point of this modification is that the reaction mixture contains sodium-dodecyl-sulphate (SDS), in order to separate lipids from the proteins. The original Lowry method gave lower protein contents in the insoluble fractions than the modified one, indicating that some lipids may remain attached to the proteins still in the alkali solution. All the measurements from one tissue were made in three parallels and the average was taken as a result characterizing that animal. Known concentrations of bovine serum albumin were processed as the tissue proteins and gave a calibration curve for quantification. Protein contents are given in mg protein per g wet weight of the tissue. WIP is also given in percents of total proteins (Tables 1 and 2). At the beginning of the experiments, total protein contents were also measured before separating the two fractions. Since, however, the results agreed well with the sum of the WIP and WSP, this step was omitted later on.

In vitro *tests*

In order to estimate the really cross-linked portion of the WIP, the following *in vitro* tests were carried out:

(1) The tissue homogenate was heat-treated by keeping at 64°C for 10 min. This temperature was sufficient to solubilize the non-crosslinked part of the collagen of rat tail tendons (Verzár, 1956, 1964, etc.). The separation of WIP was performed thereafter as described above.

(2) Urea was added to the homogenate before the separation took place to a final concentration of 3 M. Protein aggregates due to formation of weak bonds like H-bridges, etc. are disrupted by this concentration of urea (Roubal and Tappel, 1966). The WIP was separated after several hours.

(3) Protease inhibitors were added to the homogenates, in order to eliminate any eventual effect of such enzymes on the WIP percents. We used *N*-ethyl-maleinimide (NEM) in 10^{-4} M final concentration. This is an SH-blocking inhibitor. The other protease-inhibitor used was GORDOX® (G. Richter, Budapest). This compound inhibits the protease of pancreatic type (used also for human therapy). It was applied in a concentration of 0.5% corresponding to 50 units of kallikrein-inactivator per ml. The effect of heat-treatment was also tested in homogenates containing protease inhibitors.

Centrophenoxine treatment

Total protein as well as WIP–WSP contents were determined also in female CFY rats. First we studied the brain and liver of 15 young animals, then compared 6 old controls to 5 centrophenoxine (CPH)-treated rats. The controls and treated rats were randomly selected. CPH was administered intraperitoneally in aqueous solution in form of Helfergin® kindly provided by PROMONTA, Hamburg. Injections were given daily in a dose of 100 mg/kg body weight except Sundays for four weeks. The controls received no treatment. The age of this old group was 24 months at the beginning of the experiment and controls as well as treated rats were killed together one month later. No *in vitro* treatments were carried out in females.

Statistical comparison of the results obtained was performed using the Student's *t* test.

TABLE 1. TOTAL PROTEIN CONTENT AND WATER-INSOLUBLE FRACTIONS (WIP) OF THE BRAIN CORTEX OF MALE CFY RATS EXPRESSED IN mg PROTEIN PER g WET WEIGHT (MEANS OF FIVE ANIMALS ± S.E.M.).

Age group (months)	Total protein content	WIP	WIP after *in vitro* treatments: 64°C 10 min	NEM 10^{-4} M	NEM + 64°C 10 min	GORDOX 0.5%	GORDOX + 64°C 10 min	Urea 3 M
Young (2)	98.3 ±3.3	23.7 ±0.7 (24.1)	15.3 ±2.3 (15.6)	25.2 ±0.7 (25.6)	18.2 ±2.2 (18.5)	25.2 ±0.7 (25.5)	21.2 ±1.4 (21.6)	11.7 ±0.9 (11.9)
Significance between young and adult, $p <$	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	0.01
Adult (15)	100.9 ±1.1	23.6 ±0.4 (23.4)	20.1 ±0.9 (20.0)	24.8 ±0.3 (24.6)	23.1 ±0.7 (22.9)	25.5 ±0.4 (25.3)	23.8 ±0.8 (23.6)	17.7 ±1.3 (17.6)
Significance between adult and old, $p <$	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Old (30)	164.9 ±4.6	55.2 ±0.9 (33.5)	46.5 ±4.0 (28.2)	59.6 ±2.3 (36.2)	57.3 ±1.7 (34.8)	59.5 ±3.1 (36.1)	57.5 ±2.8 (34.9)	38.2 ±0.2 (23.2)
Significance between young and old, $p <$	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

The numbers in parentheses represent percents of total proteins.

TABLE 2. TOTAL PROTEIN CONTENT AND WATER-INSOLUBLE FRACTIONS (WIP) OF THE LIVER OF MALE CFY RATS EXPRESSED IN mg PROTEIN PER g WET WEIGHT (MEANS OF FIVE ANIMALS ± S.E.M.)

Age group (months)	Total protein content	WIP	WIP after *in vitro* treatments: 64°C 10 min	NEM 10^{-4} M	NEM + 64°C 10 min	GORDOX 0.5%	GORDOX + 64°C 10 min	Urea 3 M
Young (2)	139.8 ±3.1	38.4 ±0.9 (27.5)	34.3 ±2.8 (24.6)	39.7 ±1.3 (28.4)	38.3 ±0.8 (27.4)	40.0 ±1.2 (28.6)	35.8 ±2.8 (25.6)	20.3 ±3.0 (14.5)
Significance between young and adult, $p <$	0.01	0.001	N.S.	0.001	0.01	0.01	0.01	0.001
Adult (15)	157.4 ±2.8	46.6 ±0.6 (26.6)	41.7 ±1.6 (26.5)	49.5 ±1.0 (31.4)	46.6 ±1.6 (29.6)	48.6 ±1.5 (30.9)	46.4 ±1.3 (29.5)	37.2 ±1.6 (23.6)
Significance between adult and old, $p <$	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.01
Old (30)	205.1 ±4.9	79.0 ±2.0 (38.5)	74.7 ±2.4 (36.4)	81.8 ±2.6 (39.9)	77.7 ±4.7 (37.9)	83.9 ±2.6 (40.9)	78.3 ±4.9 (38.2)	55.5 ±4.7 (27.1)
Significance between young and old, $p <$	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

The numbers in parentheses represent percents of total proteins.

RESULTS

Electron microscopy

As it could be expected, the homogenization of tissues in water resulted in a complete destruction of the cellular and subcellular structures. Since the salt content of the tissues was diluted 100-fold under the given conditions, the cell components were exposed to a very strong osmotic shock. Accordingly, one finds only membrane fragments and some granular structures in both the WIP and WSP fractions (Fig. 1). It seems to be evident that no tissue specific structures remained in either of the fractions. Even the myelin fragments can be seen in both kind of preparations of the brain, however, one has the impression that the myelin fractions of the WIP pellet are larger than those in the WSP. It seems to be justified to assume on the basis of the ultrastructure of the fractions that the separation took place according to the actual size and density of the subcellular fragments. The membraneous elements which could not be dispersed into sufficiently small particles under the given conditions could not remain in the supernatant of the homogenate. The term "water-solubility" in this case involves rather the fragility of the cellular components under the given conditions than true solubility in a physico-chemical sense.

Vol. 16 1981

Chemical determinations

The results of protein determinations carried out on the brain and liver of male rats are summarized in Tables 1 and 2. The total protein content of the brain tissue was identical in the young and adult animals, whereas in the old group there was a considerable increase. The WIP fraction showed similar behaviour: the old brains contain more than twice as much WIP as compared to the young and adult groups. Percentage values of WIP are also significantly higher in the old animals. As regards the *in vitro* treatments, the mild heat treatment at 64°C solubilized a considerable part of WIP, however, in the old rats the heat-treated WIP was three times higher than that of the young animals. Both types of protease inhibitors increased the WIP only slightly indicating a negligible role of proteases in the actual WIP:WSP proportions. It is noteworthy that heat-treatment in the presence of protease-inhibitors reduced the WIP amounts to a lesser extent than heat-treatment without them in the young group. However, it had no influence on WIP in adult and old animals. The young and adult brains differ significantly from each other only in one parameter, namely, in the concentration of proteins being insoluble even in the presence of 3 M urea. The adult brains contain 151 and the old brains 326% of this kind of protein found in the young brains (Table 1).

Similar tendencies were observed in the case of liver (Table 2). Nevertheless, the liver of adult animals shows already significant differences as compared to the young ones. The only exception is the amount of WIP after heat-treatment, where no significant difference was found between the young and adult livers. Nevertheless, the increasing tendency of this parameter is also clear. The liver WIP values were less influenced by the heat-treatment and the protease inhibitors, as compared to the brain. The urea-insoluble fraction represented almost the same percent of total proteins of the liver as in the brain, however, in absolute protein concentration it was much higher. Ageing caused an increase of urea-insoluble proteins of the liver: 183 and 273% of the young value was found in the adult and old groups, respectively (Table 2).

Table 3 shows the total protein and WIP contents obtained in female rats. It is evident that the tendencies of these parameters are similar as shown in the males. It should be

TABLE 3. TOTAL PROTEIN CONTENTS AND WATER INSOLUBLE FRACTIONS (WIP) IN THE BRAIN AND LIVER OF YOUNG, OLD AND OLD-CPH-TREATED FEMALE CFY RATS, EXPRESSED IN mg PROTEIN PER g WET WEIGHT (MEANS ± S.E.M.)

		Brain		Liver	
Age group (months)	Number of rats	Total protein	WIP	Total protein	WIP
Young (1–4)	15	94.2 ±2.1	22.0 ±1.0 (23.4)	141.2 ±1.9	37.1 ±1.1 (26.3)
Old (25)	6	135.3 ±4.9	53.2 ±2.0 (39.3)	179.6 ±3.9	54.4 ±1.4 (30.3)
Significance between young and old, $p <$		0.001	0.001	0.001	0.001
Old-CPH-treated (25)	5	122.7 ±3.8	40.4 ±1.5 (32.9)	168.3 ±4.5	42.1 ±0.8 (25.0)
Significance between old and old-CPH-treated, $p <$		N.S.	0.001	N.S.	0.001

Numbers in parentheses are percents of total proteins.

noted that the young group contains animals of 1–4 months of age. These have been pooled together because no significant differences were obtained when animals of different ages in the range were studied separately. CPH-treatment of four weeks resulted in a decrease of both the total and WIP concentrations in both organs studied (Table 3). Although the differences in the total protein content were not significant at the given level of sampling, the WIP fractions were significantly lower in both organs of the treated animals.

DISCUSSION

First we should like to comment on the reliability of the method used for protein determinations. Evidently, the Folin color reaction can only be accepted for quantification of proteins, if the results obtained are comparable with those based on Kjeldahl N-determinations. As regards the brain tissue, the Kjeldahl method gave 117 and 118 mg protein per g wet weight in young (2–4 months of age) rats and rabbits, respectively (Medvedeva, 1937; Lowry *et al.*, 1951), if no separation of proteins from lipids was carried out. Lowry *et al.* (1951) obtained in unpurified rabbit brain 98 mg protein/g wet weight by the Folin color reaction which is in complete agreement with our results. The Kjeldahl method gives about 20% higher values in the unpurified tissue extracts due to other than protein sources of N (Lowry *et al.*, 1951). If we consider this percentage, our results obtained in adult and old groups are also completely comparable with those of Medvedeva (1937) describing 116 and 142 mg protein/g wet weight for the adult (6–12 months) and old (about 2 years) rats, respectively. The results obtained by us in the liver are also comparable with the young, adult and old values of Medvedeva (1937), although somewhat lower than that of the rabbit liver given by Lowry *et al.* (1951). This latter difference, however, may reflect some species differences.

It is clear from the above comparison that the method used by us for protein determination is reliable in so far as it explores all the proteins present in both the young and old tissues. The second conclusion to be drawn is that protein determinations giving unrealistically low protein content like 50 mg/g wet weight of the brain (Naber *et al.*, 1979) should be

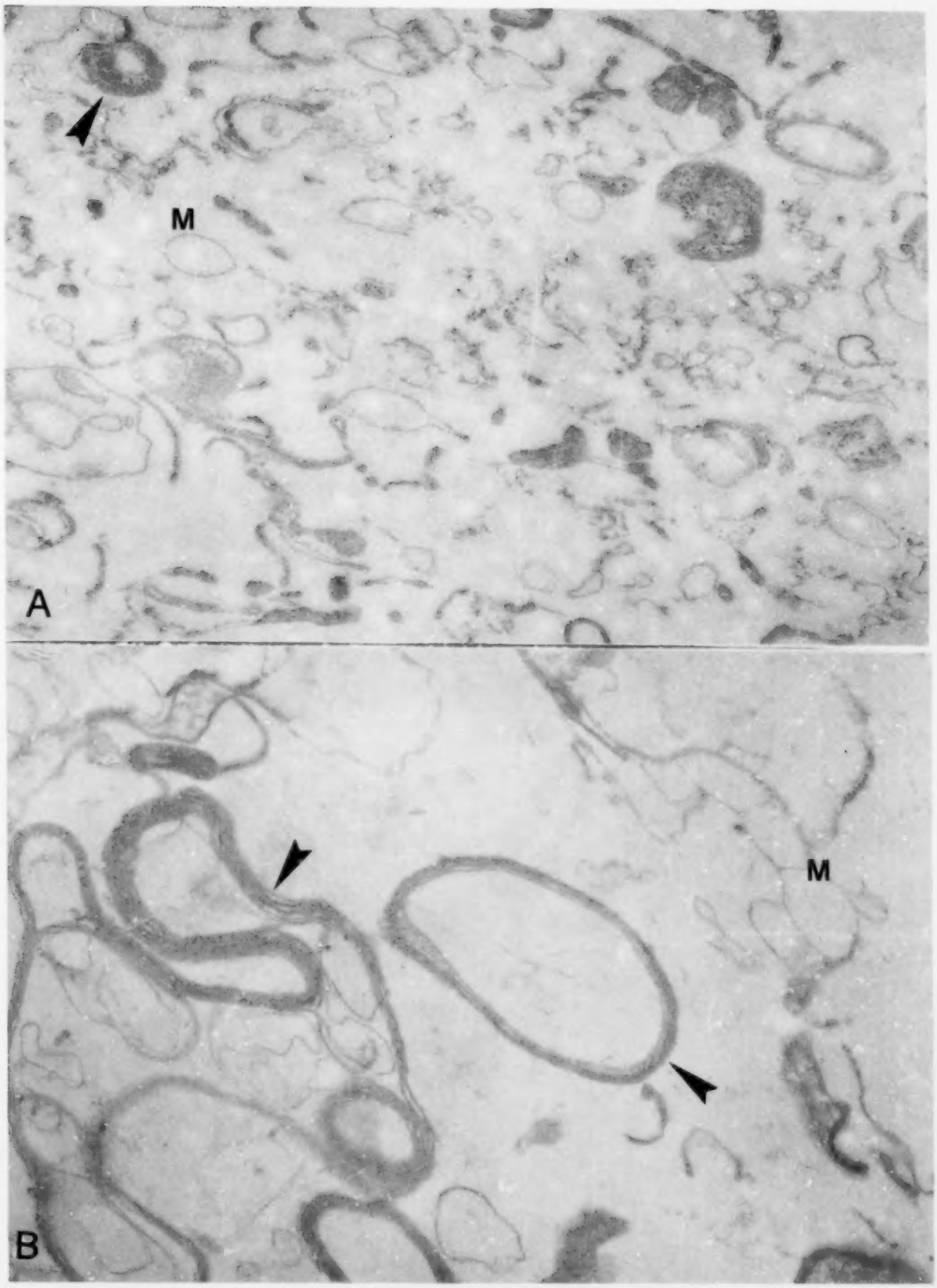

FIG. 1. Electron micrographs of the WSP (A) and the WIP (B) fractions of the brain cortex of an adult animal. Note that the cellular organelles are completely destroyed in both fractions. The whole picture is of poor contrast due to the osmotic shock. Only "microsome"-like vesicular formations (M) can be seen in various sizes together with membrane fragments and some granular substance. Myelin debris occurs in both fractions (arrows), however, it is more abundant and even the average size of the fragments is larger in the WIP. × 25,000.

Vol.
16
1981

Vol
1
198

revised from a methodical point of view. Furthermore, the results according to which no age-dependent change in the protein content of brain and liver tissue was observed should also be interpreted very carefully for two reasons. First, the animals may not have been old enough [e.g. Grossfeld and Shooter (1971) studied mice up to an age of 700 days which may not be sufficient to get real ageing phenomena of the brain in the given strain]. Secondly, it is possible that the method used is suitable to solubilize the proteins in the young animals but not in the old ones where much more efficient methods are necessary to explore all the proteins.

The age-dependent increase of brain and liver protein contents was supported in our laboratory by using another method as well. An X-ray microanalytic method has been elaborated recently (Zs.-Nagy *et al.*, 1981) allowing us to measure the intracellular dry mass and water contents. The application of this method to brain and liver cells of rats revealed that there is an age-dependent increase of the dry mass content and a corresponding decrease of the water content of the cells between 1 and 32 months of age. We obtained about 80 wt% intracellular water in the brain cells of 1-month-old animals decreasing to 72–73% by the age of 32 months. This means that the dry mass content increases from 20 to 27–28 wt% in the intracellular mass; therefore, the protein content might have well increased about 40 relative percent. The decrease of water content is not linearly proportional to age: it decreases more rapidly above the age of two years (Zs.-Nagy *et al.*, 1981). These results are consistent with those of Medvedeva (1937) in spite of the fact that she measured total water content of the brain tissue and we measured intracellular water concentrations.

Vol. 16 1981

The water solubility of proteins as defined by our experimental conditions also displayed an age-dependence. It should be stressed that our results must not be compared to other observations obtained using quite different experimental conditions. We consider it as an important phenomenon that the old tissues contain much more WIP than the young and adult ones.

Proteins may become less soluble in water for several reasons. Nevertheless, one can classify the possible reasons into two main groups. The first group involves weak interactions like H-bonds, Van der Waals forces, etc., resulting in some aggregation of molecules. These types of bond are easy to disrupt: mild thermal treatment or concentrated urea solutions are able to destroy them. The second group can be defined as strong interactions where the intermolecular bonds are of high energy, such as covalent crosslinks. These bonds are resistant to mild heat-treatment and even to high urea concentrations. The fact that in both tissues studied the WIP showed a considerable age-dependent increase even after heat-treatment and in 3 M urea suggests that the proteins really become crosslinked during ageing. In this respect we see a complete parallel between the ageing of collagen (Verzár, 1956, 1964, 1969, etc.) and the tissue proteins.

Although the protease inhibitors used by us showed no considerable effect on the WIP amounts, it is interesting to note that the heat-resistance of WIP increased in the presence of them. This may be due probably to direct chemical effects. For instance, it is known that NEM binds irreversibly to proteins.

The question arises; what may be the reasons for the supposed crosslinking of proteins during ageing? We believe that the primary event in this process is the formation of aggressive free-radicals during oxidative processes of the cell metabolism. As we pointed out elsewhere (Zs.-Nagy and Nagy, 1980), proteins become polymerized covalently under the influence of free radicals generated *in vitro* during peroxidation of ferrous ions.

Since the cells cannot exist without oxidations, the living systems continuously produce a certain amount of free-radicals during their whole life. Since, however, the protecting systems against the free-radical effects cannot eliminate perfectly the consequences of them, the cells are forced to replace the damaged proteins. The rates of this replacement depend on the actual state of the chromatin and the cytoplasm realizing the transcription and translation of the genetic information.

The cell can maintain its integrity until the rates of replacement of damaged proteins are sufficiently high. However, these rates are not independent from the general metabolic and functional conditions of the cells. A recently developed "membrane hypothesis of ageing" (Zs.-Nagy, 1978, 1979) predicts that there is an interrelationship of regulatory character between the passive permeability of cell membrance for potassium and the state of chromatin. Namely, if the potassium permeability decreases due to alterations of lipid and/or protein composition of the membrane, the cell accumulates potassium automatically, and this is an advantage from the point of view of cell excitability. However, the increased intracellular potassium content results in a more condensed state of chromatin involving a slowing down of the RNA-synthesis. This will reduce the rate of protein synthesis which will further destroy the membrane functions. This is a circulus vitiosus leading to a complete inability of the cell to perform its functions.

In our opinion, the effect of CPH on the WIP may be interpreted in these terms. It is known that the effective part of the CPH molecule is dimethylamino-ethanol (DMAE), and this compound was found to form phosphatydyl-DMAE in the brain cell membranes (Miyazaki *et al.*, 1976). On the other hand, DMAE proved to be an effective free-radical scavenger, strongly protecting the protein molecules against free-radical attacks (Zs.-Nagy and Nagy, 1980). CPH-treatment resulted in a decrease of the intracellular potassium content of the cortical nerve cells of old rats (Zs.-Nagy *et al.*, 1979). In the light of these data we believe that CPH acts primarily on the cell membrane by improving its passive potassium permeability. As a consequence, the intracellular potassium concentration decreases, the cell increases the rate of protein synthesis. This results in an improvement in the elimination of the damaged components: in fact, the lipofuscin content of neurons and myocardium significantly decreases under CPH treatment (Zs.-Nagy *et al.*, 1979). What is more, the animals survive longer and with better physiological learning capacities due to CPH-treatment (Nandy, 1978). An improvement in several synaptic morphometric parameters has also been demonstrated in the cerebellar glomerulus under the influence of CPH (Giuli *et al.*, 1980).

We are aware of the speculative character of this interpretation. Nevertheless, it seems to us a hypothesis of a certain value since it helps us to project further experiments.

SUMMARY

The cerebral cortex and liver tissues of young, adult and old (1–4, 15 and 25–30 months of age, respectively) CFY rats of both sexes were investigated. Water-soluble (WSP) and water-insoluble (WIP) protein fractions were separated after homogenization of 10 mg tissue per 1 ml of distilled water, by centrifugation at 500 ***g***. The WSP and WIP fractions were analyzed by electron microscopy and their protein contents were determined by a modified Folin phenol reaction. Electron microscopy revealed a complete destruction of the cell organelles due to the strong osmotic shock. Total protein contents of both tissues increased significantly in the old rats and also the WIP contents were much higher

in aged animals than in the young and adult ones. Mild heat treatment (64°C, 10 min) or 3 M urea were able to resolubilize a considerable portion of the WIP in the young animals, however, in adults and olds their effect was strongly reduced. Protease inhibitors displayed only a very limited influences on the WIP. The conclusion was reached that a considerable portion of WIP of the old animals may be covalently cross-linked. Certain methodical problems of protein determination are also discussed. *In vivo* application of centrophenoxine (100 mg/kg body weight) for 4 weeks significantly reduced the WIP content in 25-month-old females, and the total protein content also displayed a decreasing tendency. The results are discussed in terms of the membrane hypothesis of ageing.

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Vol. 16 1981

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Vol
16
198

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EFFECT OF VARIED DIET, RESTRICTED FEEDING AND STARVATION ON THE CHARACTERISTICS OF COLLAGEN OF YOUNG GARDEN LIZARDS

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Vol.
16
1981

INTRODUCTION

THE RATE of growth and ageing in animals can be manipulated by using various parameters. Food regimes, temperature and chemicals are some such factors. Earlier, attempts have been made to show that these factors influence the ageing process in insects, fish and mammals (Comfort, 1964; Kohn, 1978; Timiras, 1972). Even though there are some reports of studies on factors influencing the morphometric growth, information so far on biochemical aspects of growth and ageing in reptiles is inadequate (Patnaik, 1980). Recently we have reported (Brahma *et al.*, 1981) that the mammalian prolactin acts as an anticross-linking agent and TSH as a cross-link promotant in collagen of young male garden lizards. Porcine STH was also shown to accelerate collagen ageing in young male garden lizards (Panigrahy and Patnaik, 1975). As assessment of collagen ageing has been well documented as correlating with physiological ageing of vertebrates it was considered worthwhile to extend the study of manipulating factors, other than chemicals, to reptiles.

The present study concerns the influence of nutritional status such as varied diet, restricted feeding and starvation in young male garden lizards.

MATERIALS AND METHODS

Young male garden lizards (*Calotes versicolor*) weighing between 7 and 15 g and having a snout-to-vent length of 62–87 mm were collected from Berhampur localities during November–March. Before use, the animals were maintained in the laboratory for about a week on a diet of goat liver on alternate days and tap water was provided *ad libitum*.

Varied diet

The control group of lizards (10) was fed with goat liver on alternate days and the experimental group of lizards (10) was provided with live earthworms and grasshoppers on alternate days. Tap water was provided to both animal groups *ad libitum*. The experiment was carried out for 3 weeks and on the 22nd day the lizards were killed by a blow on the head.

Weighed quantities of long bones, dorsal skin and tendon from tail and phalanges were collected from control and experimental animals. Salt soluble collagen was extracted in 0.15 M sodium chloride (NaCl) for 24 h at 8°C. The salt soluble and salt insoluble fractions were separated by centrifugation at 1500 r.p.m. in a Remi centrifuge. Acid soluble collagen was extracted in 0.45 M acetic acid (24 h at 8°C) from salt insoluble fractions. Acid soluble and acid insoluble fractions were separated by centrifugation at 1500 r.p.m. for 15 min (tendon samples were separated by centrifugation at 7000 r.p.m. for 15 min in a K– 24 refrigerated MLW centrifuge).

Salt soluble, acid soluble and acid insoluble fractions were hydrolysed in 6 N HCl in sealed tubes at 110°C for 16 h. The hydroxyproline contents of the neutralised samples were estimated following the method of Neuman and Logan (1950) as modified by Leach (1960). Values for collagen content were derived from the hydroxyproline values by multiplying by 7.46.

Restricted feeding

The control group of animals (12) was fed with goat liver on alternate days whereas the experimental group of lizards (15) was fed on every fourth day. Tap water was provided to both animal groups *ad libitum*. With

this feeding schedule, the experiment was continued for a period of 21 days and on the 22nd day the animals were killed by a blow on the head.

The collagen contents of bone, skin and tendon were estimated as described earlier.

Starvation

While the control group of lizards (16) was fed with goat liver on alternate days, the experimental group of lizards (17) was subjected to starvation stress for a period of 3 weeks, but with provision for drinking water. Lizards belonging to control and experimental groups were killed on the 22nd day and the collagen contents of bone, skin and tendon were estimated as described earlier.

RESULTS

Varied diet

Following consumption of varied diet the percent of solubility of collagen in salt medium increased in skin at 0.05 confidence level and in tendon at 0.01 level, but no significant change occurred in bone (Fig. 1).

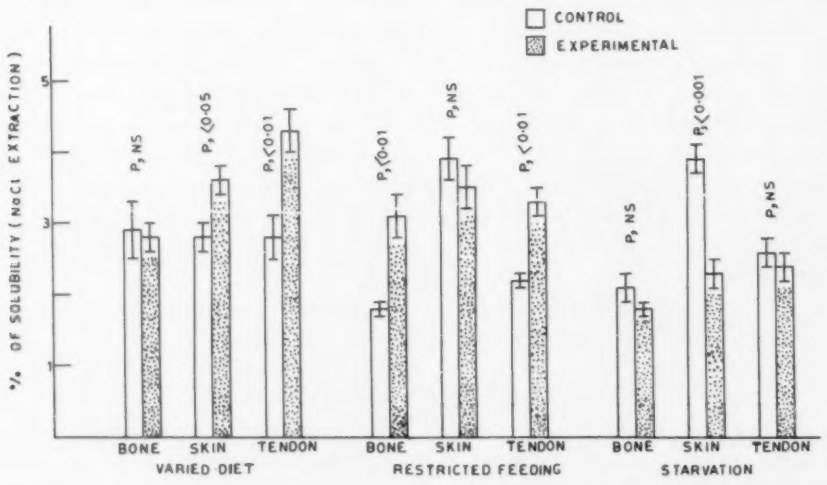

FIG. 1. Effect of varied diet, restricted feeding and starvation on the solubility (in salt medium) of collagen in bone, skin and tendon.

Similarly, the salt soluble/salt insoluble collagen ratio increased significantly in skin and tendon at 0.01 confidence level (Fig. 2).

The percent of solubility of collagen in acid medium and the acid soluble/acid insoluble collagen ratio in skin decreased at 0.05 confidence level, but not in bone and tendon (Figs. 3 and 4).

The total collagen content increased significantly in skin at 0.001 confidence level and decreased in tendon at 0.05 confidence level (Fig. 4).

Restricted feeding

Such a feeding schedule in young male garden lizards resulted in an increase in percent of salt solubility of collagen in bone and tendon at 0.01 confidence level, but not in skin (Fig. 1).

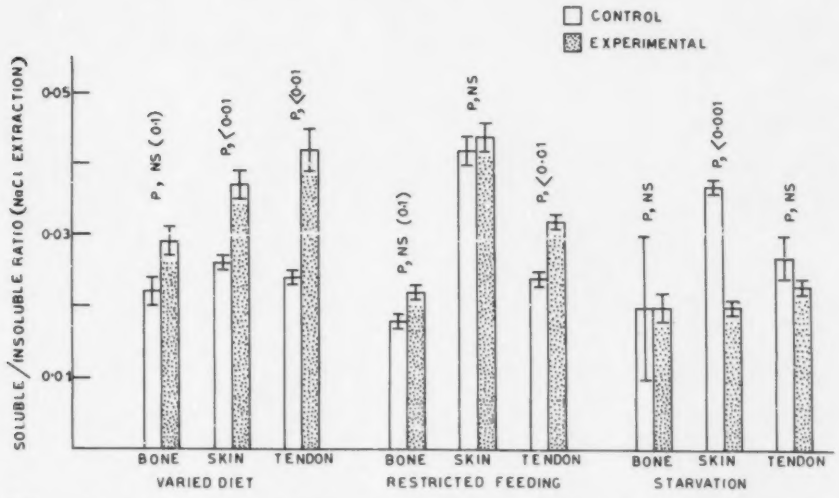

FIG. 2. Effect of varied diet, restricted feeding and starvation on the soluble/insoluble collagen ratio (in salt medium) in bone, skin and tendon.

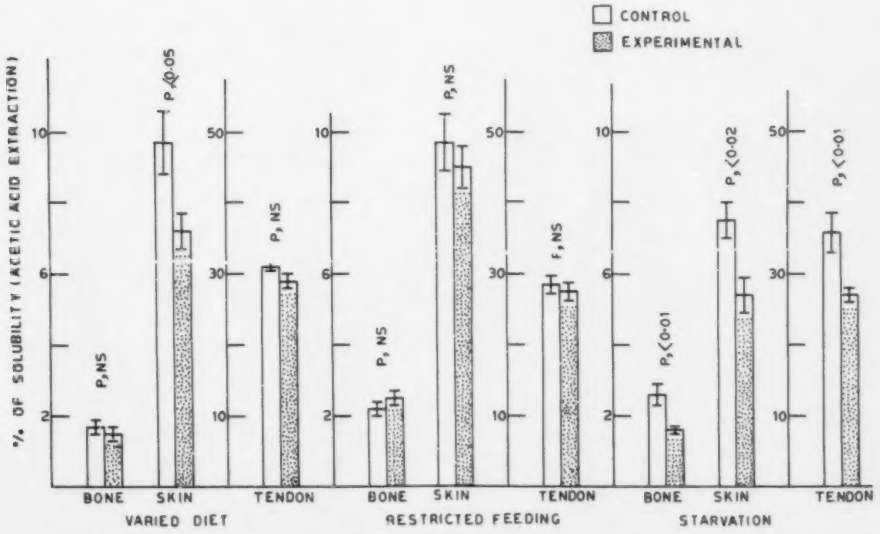

FIG. 3. Effect of varied diet, restricted feeding and starvation on the solubility (in acid medium) of collagen in bone, skin and tendon.

Similarly, the ratio of salt soluble/salt insoluble collagen increased significantly in tendon at 0.01 confidence level (Fig. 2).

None of the characteristics of collagen extracted in acid medium showed a significant change following restricted feeding (Figs. 3, 4 and 5).

Starvation

Following a starvation period of 3 weeks, the percent of solubility of collagen in salt medium and the salt soluble/salt insoluble collagen ratio in skin decreased at 0.001

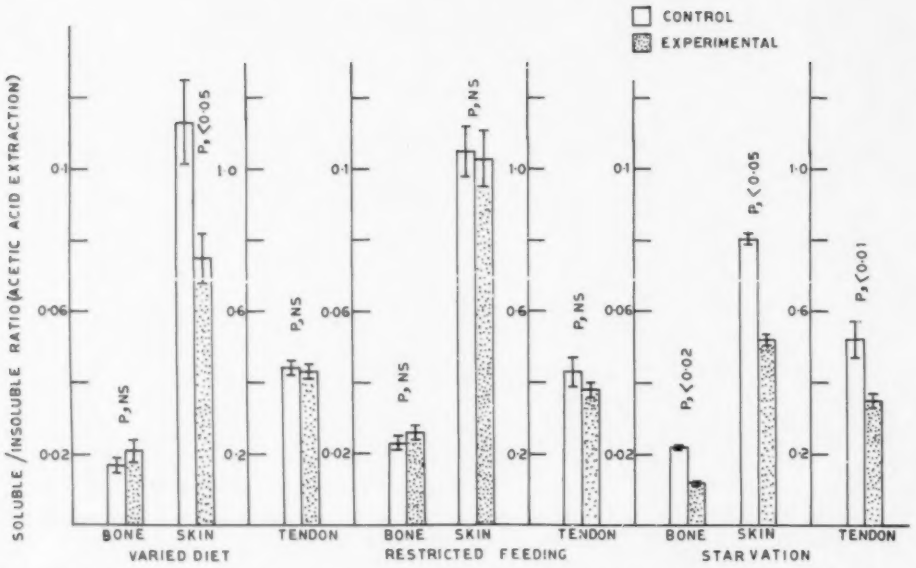

FIG. 4. Effect of varied diet, restricted feeding and starvation on the soluble/insoluble ratio (in acid medium) of collagen in bone, skin and tendon.

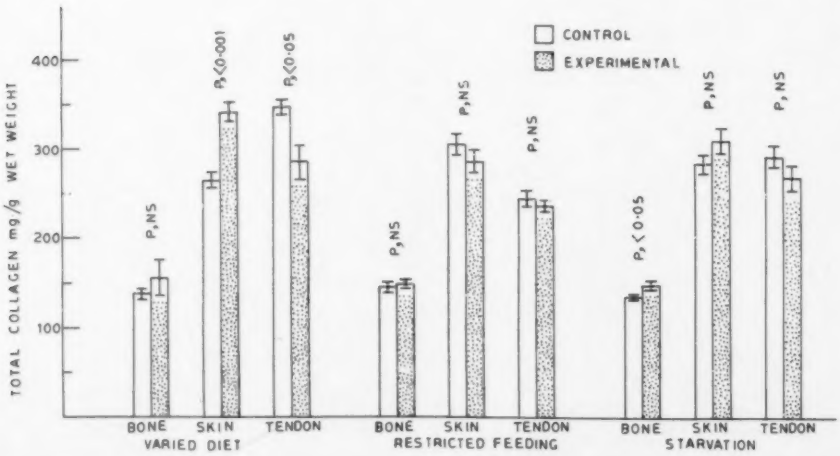

FIG. 5. Effect of varied diet, restricted feeding and starvation on the total collagen contents of bone, skin and tendon.

confidence level, but no significant change occurred in bone and tendon (Figs. 1 and 2).

Similarly, the percent of solubility of collagen in acid medium and the ratio of acid soluble/acid insoluble collagen decreased significantly in bone, skin and tendon (Figs. 3 and 4).

The total collagen content increased significantly in bone at 0.05 confidence level, but not in skin and tendon (Fig. 5).

DISCUSSION

Neutral salt soluble collagen extracted in 0.15–0.45 M NaCl is more dependent on growth rate of animals whereas acetic acid soluble collagen is more dependent on ageing. Moreover, since the metabolic status of the individual tissue differs from one to the other as do the rates of growth and ageing, the response to manipulating factors may not be similar. Results of the present study need to be considered keeping the above points in mind.

The ageing of tail tendon collagen, as measured by the breaking time in 7 M urea at 40°C, was retarded by food restriction in male rats (Everitt, 1971) but above this level the excess of food accelerated the ageing. The ageing of collagen fibres in rat tail tendon slowed down in underfed rats (Chvápil and Hrůza, 1959; Hrůza and Hlávacková, 1969). That this effect is more evident in young animals was suggested by Sovjtkova *et al.* (1972). Our results, that the salt solubility of collagen in garden lizards increases upon food restriction, confirms that such a control prevents cross-link formation of collagen and accelerates the growth rate.

Vol. 16 1981

In another set of experiments, the control group of lizards was fed with goat liver whereas the experimental group of lizards was allowed to consume their natural varied diet (live grasshoppers and earthworms) freely. This led to the prevention of cross-link formation of collagen in skin and tendon as revealed by the salt extraction method. However, the acid extraction method showed accelerated ageing of collagen in skin. This contrasting picture in skin, that varied diet favours increased rate of growth and also accelerates ageing, cannot be resolved from the present findings and needs to be elaborated in future studies. The analysis of the composition of food might provide some useful explanation as a high fat diet was shown to increase collagen stability (Sovjtkova *et al.*, 1972).

The effect of starvation stress on ageing of animals has not received due coverage (Bellamy, 1973). Even though food restriction retards ageing, excess of feeding and complete starvation show a reverse affect (Everitt, 1971; Everitt and Porter, 1976). Our results, that the salt solubility and the acid solubility of tissue collagen decline following a starvation period of 3 weeks, suggest that this physiological stress accelerates cross-link formation in collagen, thereby giving an indication of retardation of growth and the acceleration of the ageing process.

The changes in total collagen content of tissues under various experimental conditions were not consistent and hence no definite conclusion could be drawn.

Thus, it appears that as in other groups of animals (insects, fish and mammals) the nutritional status has a profound influence on the growth and ageing of the terrestrial reptile, *Calotes versicolor,* as revealed by the characteristics of collagen. Further studies on the feeding of different types of food to lizards maintained in the laboratory and comparing the results with those of lizards having a natural diet in the wild are in progress to obtain detailed information on the role of food factors on the growth and ageing of reptiles.

SUMMARY

Attempts have been made to correlate the nutritional status of garden lizards with the growth and ageing process taking the characteristics of collagen as the parameter. Feeding of a varied diet results in prevention of cross-link formation in skin and tendon as revealed by the increase in salt solubility of collagen. A similar trend was observed in the

bone and tendon of garden lizards subject to food restriction. These results suggest that both a varied diet and restricted feeding increase the rate of growth. Starvation stress for a period of 3 weeks led to a decrease in both salt solubility of skin collagen and acid solubility of bone, skin and tendon collagen, thereby indicating a retardation of growth and an acceleration of the ageing process.

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AGING OF MEMBRANE TRANSPORT MECHANISMS IN THE CENTRAL NERVOUS SYSTEM—2-DEOXY-D-GLUCOSE TRANSPORT IN CORTICAL SYNAPTOSOMES FROM THE LONG–EVANS RAT

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Vol.
16
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INTRODUCTION

A DECLINE in nervous system function is likely to play a key role in the loss of homeostatic regulation which accompanies aging (Franks, 1970; Sohal and Sharma, 1972; Hayflick, 1976; Diamond, 1978; Lytle and Altar, 1979). Evidence is accumulating that changes with age in the process of synaptic transmission are contributory to this decline in nervous system regulatory capacity (Kent, 1976; Pradhan, 1980). Membrane transport processes are crucial to neuronal function, both in maintaining cellular homeostasis and in the process of synaptic transmission. Related to this dual role, there appear to be two types of carrier-mediated mechanisms by which organic substrates are taken up in the nervous system. On the basis of the apparent Michaelis–Menten constant (K_t) for uptake, these mechanisms are classified as either high or low affinity.

High affinity mechanisms are characterized by K_t's lower than about 5×10^{-5} M and seem to be found only for those substances which are thought to play a role in synaptic transmission; these substances are taken up by both low and high affinity mechanisms (Krnjevic, 1974; Wheeler, 1980*a*). Transport mechanisms are critical to synaptic function, both in concentrating transmitter or precursors in pre-synaptic terminals and in inactivation of released transmitter by pre-synaptic reuptake.

Low affinity mechanisms are characterized by K_t's on the order of 10^{-4} M or above (Smith, 1967; Blasberg, 1968). Low affinity mechanisms are thought to be unrelated to transmitter function, but are necessary to maintain cellular homeostasis in all types of cells. Maintenance of the proper ionic environment and procurement of substrates for energy metabolism and protein synthesis are accomplished by the operation of such low affinity transport mechanisms. Thus, alteration of transport function might be expected to have two effects in the nervous system: (1) loss of the ability of individual cells to maintain homeostasis and (2) alteration of the ability to communicate among cells.

Previous studies in this laboratory have confirmed the hypothesis that alterations in high affinity transport mechanisms accompany aging. Studies of the sodium dependent, high affinity transport of glutamic acid (Wheeler, 1980*b*) have shown that although the basic mechanism by which carrier, sodium and substrate interact in the process of transport does not change, there are substantial quantitative changes with age. As a consequence of these changes, there is a reduced capacity for uptake of substrate at any given sodium and substrate concentration.

In spite of the importance of low affinity transport mechanisms, such as that for glucose, to nervous system function, no studies of the aging of these mechanisms have been made. Glucose is the primary substrate for energy metabolism in brain tissue (Bachelard and

McIlwain, 1969). Both glucose and its analog, 2-deoxy-D-glucose (2-DG), are taken up in brain tissue by a common saturable mechanism (Joanny *et al.*, 1969; Bachelard, 1971). This report presents the results of a comparative study of 2-DG transport in cortical synaptosomes from 2- and 30-month-old Long–Evans rats.

MATERIALS AND METHODS

Male Long–Evans rats 2–3 or 29–30 months old were used in this study. These rats were housed two per cage under conditions of constant temperature and controlled illumination, with *ad lib.* access to food and water. The diet was obtained from Wayne Feeds (Allied Mills, Inc., Libertyville, Ill.) and contained a minimum of 24% protein, 4.5% fiber and 4% fat.

An extensive study of the suitability for membrane transport studies of the preparation and techniques used in the present study has been published (Wheeler, 1978). The methods described below evolved from this study.

Preparation of synaptosomes

Adult male Long–Evans rats were used in all experiments. Following decapitation with a guillotine, the brain was removed and placed on a filter paper moistened with Krebs–Henseleit solution (Lockwood, 1961), which was kept cold by placing on a Petri dish filled with ice. The cortex was rapidly dissected free, weighed and placed in 20 volumes of ice-cold 0.32 M sucrose in a 15-ml Potter–Elvehjem mortar fitted with a Teflon pestle. The clearance between mortar and pestle was 0.004–0.006 in. The homogenizer was placed in an ice water bath and the tissue homogenized for 1 min, utilizing six 10-s strokes with a speed of 840 rev/min. The homogenate was centrifuged for 10 min at 1085 ***g*** at a temperature of 2°C to remove nuclei and cellular debris. The supernatant fluid was removed and centrifuged at 27,000 ***g*** for 15 min to sediment the synaptosomes. The supernatant fluid from this procedure was decanted and discarded, the synaptosomes were resuspended in 20 volumes of ice-cold 0.32 M sucrose and allowed to stand for 30 min in the ice bath prior to the beginning of incubation.

Measurement of uptake

The uptake of ^{3}H labeled 2-deoxy-D-glucose (2-DG) was measured by incubation of synaptosomes for 4 min in a modified Krebs–Henseleit solution at 30°C. 2-DG is metabolized to 2-DG-PO_4. However, Diamond and Fishman (1973*a, b, c*) have shown that uptake, not phosphorylation, is rate-limiting and that phosphorylation is not required for uptake. They therefore conclude that the kinetic constants determined by uptake of label are representative of transport and not of metabolism. In any event, if one keeps the uptake time to a minimum so that initial velocity is truly approximated, uptake of label may be used to approximate initial velocity of transport. Therefore, 4-min uptakes were used in all experiments, rather than 15-min uptakes which are often used. 2-deoxy-D-glucose was obtained from Sigma Chemical Co., St. Louis, Missouri. All incubations were carried out in a Dubnoff metabolic shaker. The modified Krebs–Henseleit used consisted of 127.2 mM NaCl, 5.0 mM KCl, 2.7 mM $CaCl_2$, 1.3 mM $MgSO_4$, and 25 mM Tris buffer at a pH of 7.4. The concentration of 2-DG in the final incubation medium was 5.0, 2.0, 1.0, 0.6667 or 0.5×10^{-4} M. For each sodium concentration used, a solution containing the highest sugar concentration was prepared with a specific activity of 1×10^7 μCi/mol and serially diluted; thus a constant specific activity was maintained at all sugar concentrations. Concentration of sodium in the incubation medium was varied by replacement of NaCl with equiosmolar Tris buffer; 0, 19.0, 27.0, 43.0 and 120.8 mM sodium solutions were used with each of the above 2-DG concentrations. Incubation was begun by pipetting 0.2 ml of the synaptosomal suspension (∼ 10 mg of cortical tissue) into a scintillation vial containing 3.8 ml of incubation medium.

Following incubation, synaptosomes were separated from incubation medium by rapid filtration, utilizing a Millipore 30-place manifold and Millipore filters (HAWPO2500). The synaptosomes were then washed with two 5-ml aliquots of Krebs–Henseleit ([Na] = 0) at 30°C. Previous studies indicate that such a wash procedure results in little loss of label from within the synaptosomes (Wheeler, 1978). Following completion of all incubations, each filter was removed, placed in a scintillation vial and 1 ml of distilled water added. The vials were left for 1 h, then scintillation fluid was added; 24 h later, the vials were counted for radioactivity in a Packard 3320 liquid scintillation spectrometer until the error was less than 1% at the 95% confidence level. Disintegrations per minute were calculated from counts per minute by use of an external standard to determine counting efficiency.

Initial velocities for each of the 2-DG concentrations with each of the several sodium concentrations were measured with synaptosomes from a single preparation of tissue from one animal. The measurements were then repeated on additional animals.

Blanks

In order to correct for filter retention of label when incubation medium is poured through them, blanks were run with each experimental solution. A 3.8-ml aliquot of incubation medium was added to a well of the

filtration manifold, followed by the usual wash procedure. This was done with each experimental solution in the same order and with the same temporal pattern that was used for initial velocity measurements. Eighteen to twenty replications were made for each [2-DG]. The amount of 2-DG retained by the filter is unaffected by [Na], but linearly related to the 2-DG concentration. Therefore, the data were fitted to a straight line by linear regression and predicted values from this fit were determined and used as estimates of filter retention of 2-DG. Initial velocity measurements were corrected by subtracting this blank value.

Intra-synaptosomal water content

Distributions of [^{14}C]polyethylene glycol (PEG) and [^{3}H]D-galactose were utilized to compute synaptosomal water content. Synaptosomes were prepared as usual. Following removal of the supernate from the first centrifugation, sufficient PEG and galactose were added to bring the concentration of each to 10^{-4} M, including 0.05 μCi/ml PEG and 0.2 μCi/ml D-galactose. After standing for 2 h in an ice bath, synaptosomes were pelleted as usual. The supernatant fluid was sampled and counted for both carbon-14 and tritium. The remaining supernate was then removed and 5 ml of distilled water added to the pellet, vortexed and allowed to stand for 1 h. The preparation was centrifuged again at 27,000 **g** for 15 min and the supernatant fluid was sampled and counted for both carbon-14 and tritium. After correcting the counts for crossover, intrasynaptosomal volume was computed as the difference between the D-galactose and PEG volumes of distribution. Mean values for the 2-month group were 1.433 ± 0.03 (S.E.M.) μl/10 mg tissue weight compared to 1.467 ± 0.04 for the aged group. Thus, there is no significant difference between the two groups with regard to synaptosomal volume; therefore, the data were not corrected for water content, but were expressed per 10 mg tissue wet weight.

Computer optimization

In order to fit the data to the velocity equations for the various models, a parameter optimization approach was used to minimize the following function:

$$J = \sum_{i=1}^{n} \left(\frac{v_{\text{model}} - v_{\text{data}}}{v_{\text{data}}} \right)^2 .$$

J, the objective function, is the sum of the squared fractional error between the velocities of uptake predicted by the models and those observed experimentally. The parameter optimization algorithm used in this research was based on the Flexible Polyhedron approach of Nelder and Mead (1965). The algorithm was coded in BASIC and run on either a Challenger II or III Microcomputer (Ohio Scientific Instruments) fitted with 48 K bytes of 8-bit memory and a dual-drive floppy disk.

To use the BASIC program, the appropriate model equation and velocity data were first added to the optimization portion of the program. Initial guesses for all kinetic constants were then entered and program operation was begun. After the algorithm returned the optimal parameters, the program was re-started several times to insure that the minimum possible error had been achieved. Finally, the program printed the various data velocities paired with the corresponding values predicted by the equations.

RESULTS

Comparison of initial velocities

Table 1 shows a comparison of initial velocities for 2- and 30-month animals. The ratio of $v(30)/v(2)$, as a percentage, varies only from a low of 89 at a [2-DG] of 0.5×10^{-4} M and a [Na] of 0 to a high of 107 at a [2-DG] of 0.5×10^{-4} M and a [Na] of 120.8 mM. In no case is there a significant difference between the 2- and 30-month values. Thus, there has been absolutely no significant change in velocity of 2-DG uptake with age at any [2-DG].

TABLE 1. COMPARISON OF INITIAL VELOCITIES $[v(30)/v(2)] \times 100$

$S \times 10^4$ (M)	0.5	0.6	1	2	5
[Na], mM					
0	89.13	94.28	95.96	101.07	100.15
19	101.98	92.61	100.26	102.78	103.03
27	93.27	102.26	97.34	100.47	100.55
43	97.75	98.38	101.36	98.01	106.46
120.8	107.44	97.08	105.96	97.32	97.58
Mean	97.92	96.92	100.19	99.95	101.42

Vol. 16 1981

Velocity vs sodium concentration

Previous studies (Wheeler and Hollingsworth, 1979) had suggested that high [Na] might be slightly inhibitory to 2-DG uptake. However, as can be seen from Fig. 1, plots of velocity vs [Na] for all [2-DG]'s are flat for both 2- and 30-month data. Thus, [Na] has no effect on the velocity of uptake for either young or old animals.

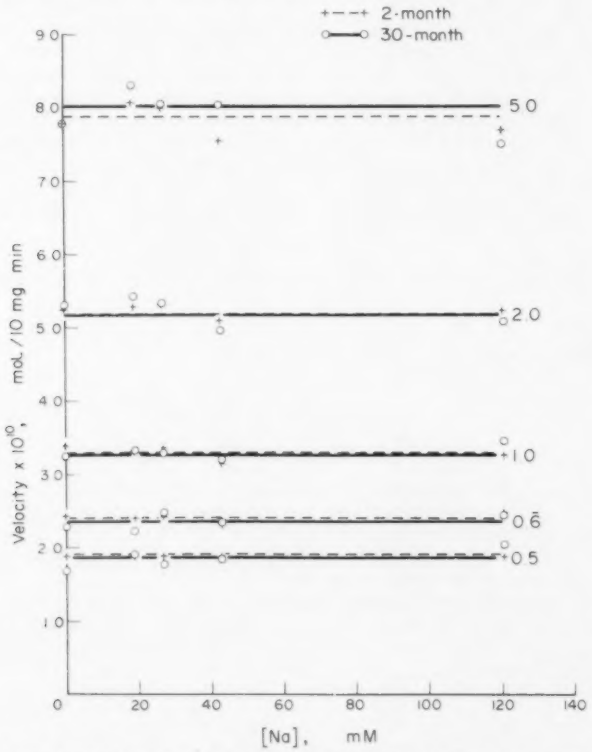

FIG. 1. Initial velocity of uptake as a function of sodium concentration. The symbols represent the mean data, while the lines represent the computed fit. 2-DG concentration $\times 10^4$ (M) is given to the right of each pair of curves.

Kinetic constants

Since [Na] had no effect on uptake, the initial velocity data for the several [Na]'s used were combined [note that $v(30)/v(2)$ now ranges between 97 and 101] and fitted to the following equation:

$$v = V_m [2\text{-DG}]/(K_t + [2\text{-DG}]) + k_d [2\text{-DG}]$$

where [2-DG] = 2-DG concentration, V_m = maximal rate of uptake; K_t = apparent Michaelis–Menten constant; k_d = diffusion constant. The fit of the data to this equation is excellent. For 2-month data, the average per cent error per data point is only 0.76 ($N = 6$), while for 30-month data, the average percent error is only 1.11 ($N = 10$). Table 2

TABLE 2. CONSTANTS GIVING BEST FIT

Constant	2-month	30-month
K_t (M)	2.6791×10^{-4}	2.8966×10^{-4}
$V_m \times 10^{10}$, mol/10 mg min	12.0831	12.6307
$K_d \times 10^{10}$, L/10 mg min	2.6873×10^{-2}	1.9694×10^{-4}

shows a comparison of the computed best fit constants for young and aged animals. For both young and aged animals, predicted k_d is so small that no measurable uptake takes place via diffusion. No appreciable changes have occurred with age in either of the other kinetic constants. K_t for 2-month animals was found to be 2.68×10^{-4} M, compared to 2.90×10^{-4} M for 30-month animals; $[K_t\,(30)/K_t\,(2)] \times 100 = 108.6\%$. Since K_t is the dissociation constant for $CS \rightleftharpoons C + S$ (C = carrier; S = 2-DG), there is no appreciable change with age in carrier affinity for 2-DG. V_m was found to be 1.21 nmol/10 mg min for 2-month animals, compared to 1.26 nmol/10 mg min for 30-month animals; $[V_m\,(30)/V_m\,(2)] \times 100 = 104.6\%$. Now $V_m = k[C]t$, in which k = translocation constant and $[C]t$ = total carrier concentration. Thus, it appears that neither the number of functioning carriers, nor the rate constant for translocation, has changed with age.

DISCUSSION

Little, if any, change occurred with age in the ability of the synaptosomes to take up 2-deoxy-D-glucose (2-DG). Since both 2-DG and glucose are taken up by the same carrier (Joanny *et al.*, 1969; Bachelard, 1971; Wheeler and Hollingsworth, 1979), this implies that the ability to take up glucose has not changed with age. Thus, aging of transport mechanisms is not a ubiquitous process common to all transport systems. Glucose is the most important substrate for energy metabolism in nervous tissue (Bachelard and McIlwain, 1969). Since aging has not altered the ability to take up this substrate, this implies that the aging cell has the capability to maintain sufficient intracellular concentrations to carry out normal metabolic functions. This has important implications concerning the ability of aging cells to maintain other homeostatic processes which depend on a source of metabolic energy.

The results of the present study are in contrast to a similar previous study of the aging of the glutamate transport mechanism (Wheeler, 1980*b*). In that study, initial velocity of uptake declined by as much as 27% (depending on the [Na] used) in 30-month compared to 2-month animals. Although the basic mechanism by which carrier, sodium and glutamate interact in the process of transport did not change with age, the kinetic constants which quantitate the mechanism were found to change with age. The most dramatic changes were seen in those constants related to maximal velocities: *V*a, the velocity of uptake at a given [Na] and infinite glutamate concentration, declined by 28% ([Na] = 140 mM), while *J*m, rate of uptake at a given [G] and infinite [Na], declined by 28–33%.

Thus, there are fundamental differences in the effects of age on 2-DG and glutamate transport. 2-DG transport is by a low affinity, sodium independent system, while glutamate transport is by a high affinity, totally sodium dependent mechanism. Low affinity transport mechanisms are common to all cells, while high affinity mechanisms are associated with those substrates thought to play a role in transmitter function. The differences seen in the effects of aging on these two types of transport mechanisms raise the question of whether the more generalized low affinity mechanisms might be more refractive to aging effects compared to the more specialized sodium dependent, high affinity systems.

SUMMARY

Initial velocity of uptake of 2-deoxy-D-glucose (2-DG) has been measured in cortical synaptosomes from 30-month-old rats and compared to results from 2-month-old animals.

Vol. 16 1981

No significant changes were found in velocity of uptake at any [Na] and [2-DG] used. The velocity data were fitted to the rate equation by use of computer optimization techniques, and the kinetic constants were estimated. The predicted diffusion constants are so low that no measurable diffusive uptake is predicted for either 2- or 30-month animals. The kinetic constants V_m (maximal rate of uptake) and K_t (Michaelis–Menten constant) were found not to change with age. The results contrast to a similar previous study of glutamate transport, in which substantial declines in carrier capability were found in the 30-month age group.

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DECLINE IN HEPATIC MICROSOMAL MONOOXYGENASE COMPONENTS IN MIDDLE-AGED FISCHER 344 RATS

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Vol. 16 1981

INTRODUCTION

THE AGING process involves physiological changes which alter an animal's response to drugs. Early studies with old rats demonstrated that the altered response could be attributed in part to a decrease in the metabolism of drugs, as demonstrated by *in vivo* and *in vitro* experiments in which the capacity of the liver microsomal drug metabolism system declined with age (Kato and Takanaka, 1968*a, b, c*). It was accepted that this age-related decline was due to decreases in components of the hepatic cytochrome P-450-dependent monooxygenase system. However, conflicting reports on the effects of aging on the microsomal enzyme system have recently appeared.

The microsomal monooxygenase system includes three major components, cytochrome P-450, NADPH–cytochrome P-450 reductase (NADPH–cytochrome c reductase) and phospholipid. Cytochrome b_5 may be involved, too. Several investigators have reported that cytochrome P-450 specific content and NADPH–cytochrome c reductase activity are decreased in liver microsomes from old rats (Kato and Takanaka, 1968*a, b*; Kao and Hudson, 1980; McMartin, O'Connor, Fasco and Kaminsky, 1980; Schmucker and Wang, 1980). An age-related reduction in the quantity of microsomal phospholipid has also been found (Birnbaum and Baird, 1978*b*; Grinna and Barber, 1972; Grinna, 1977). On the other hand, others have shown that aging does not lower cytochrome P-450 content (Baird, Nicholosi, Massie and Samis, 1975; Birnbaum and Baird, 1978*b*), cytochrome c reductase activity (Aldeman, 1971; Birnbaum and Baird, 1978*a, b*) or cytochrome b_5 content (Birnbaum and Baird, 1978*a*; McMartin, 1980) in rat liver microsomes. Furthermore, a study with senescent mice indicated that drug metabolism activities could be markedly altered (increased or decreased) while cytochrome P-450 content, reductase activity and phospholipid content remained unchanged (Birnbaum, 1980). Although some of these apparently contradictory results may be attributed to differences in animal sex, strain and species, or to differences in the ages of the "young" and "old" animals used for comparisons, it is clear that the exact mechanisms for the age-related changes in drug metabolism remain unresolved.

The transition between young adulthood and old age is important for understanding the underlying causes of aging processes. The recent studies that examined microsomal drug metabolism in middle-aged rats found no significant differences in monooxygenase activities between middle-aged and young-adult animals (Birnbaum and Baird, 1978*b*; McMartin *et al.*, 1980). In these studies the middle-aged rats were 12 months and the young-adult rats were 3 or 4 months of age.

In this study we found significant differences between middle-aged (14 months) and young-adult (3–5 months) rats in liver microsomal monooxygenase components and drug

metabolism activities. Changes in the lipid composition of the hepatic microsomal membrane were also seen. A preliminary report of these results has been presented (Rikans and Notley, 1980).

MATERIALS AND METHODS

Male Fischer 344 rats were purchased from Charles River Breeding Laboratories at 7 weeks of age and maintained in a separate room within our animal facilities for the duration of the study. The rats were given a semipurified diet (AIN 76 semipurified Diet®, Nutritional Biochemicals) and water *ad lib.* They were housed 5/cage until 6 months of age and 3/cage from 6 to 16 months of age, in heat-sterilized hard-wood bedding, under filter caps. The room in which the animals were housed was kept free of cigarette smoke, pesticides, fumigants, disinfectants, etc. Temperature, humidity and lighting remained constant.

Rats were killed 3 h after the onset of the 12 h light periods. The livers were removed and homogenized in 4 volumes of a solution containing 1.15% KCl and 0.05 M Tris–HCl, pH 7.5. The homogenates were centrifuged at 10,000 ***g*** for 15 min and the resulting supernatant fractions centrifuged at 78,000 ***g*** for 90 min. The 78,000 ***g*** pellets were washed once with homogenization medium and the final microsomal pellets suspended in 0.1 M Tris–HCl, pH 7.5, to a protein concentration of 5–10 mg/ml. All solutions were kept at 0–4°C. Centrifugations were performed at the ***g*** values determined for the average radii of the rotors used. The recovery of microsomal membranes was estimated by determining the total activity of the marker enzyme, cytochrome c reductase, in liver homogenates versus the microsomal fraction (Eriksson, DePierre and Dallner, 1978).

Cytochrome P-450 was determined in microsomes from the reduced CO difference spectrum and cytochrome b_5 from the NADH-reduced difference spectrum (Omura and Sato, 1964). Rates of cytochrome c reduction by NADPH were determined at 23°C in a high ionic strength buffer as described by Vermilion and Coon (1978). The reduction of cytochrome P-450 in microsomes was measured at 15°C by following the formation of the reduced cytochrome P-450–CO complex after NADPH addition under anaerobic conditions (Zannoni, Flynn and Lynch, 1972). Microsomal fatty acids were determined using the Folch extraction procedure and gas chromatographic analysis of fatty acid methyl esters (May and McCay, 1968). Phospholipids were determinted as inorganic phosphate in the total extracted lipid (Fiske and Subbarow, 1925). Protein was measured in the presence of sodium dodecyl sulfate by a modification (Markwell, Haas, Bieber and Tolbert, 1978) of the Lowry procedure.

The drug metabolism assays were performed using freshly-prepared microsomes. Conditions were optimized for each reaction so that activities were proportional to enzyme concentration and linear with time. Incubations were carried out in a shaking water bath at 37°C. The assay for nitroanisole *O*-demethylase activitiy was based on the procedure of Netter and Seidel (1964). Reaction mixtures, containing 0.3–0.6 mg microsomal protein, 1 mM *p*-nitroanisole, 6.8 mM glucose-6-phosphate, 0.27 mM NADP, 100 mM Tris–HCl (pH 7.8) and 1 unit of glucose-6-phosphate dehydrogenase, in a total volume of 2.0 ml, were incubated for 15 min. Reactions were stopped by adding 0.2 ml of 2.2 M NaOH, which served to clarify the mixture (Becker, Meehan and Bartholomew, 1978), and the absorbance of the product, *p*-nitrophenol, was measured at 415 nm. Aniline hydroxylase activity was determined according to Imai *et al.* (1966) except that the concentrations of aniline and nicotinamide were 1.25 and 2.5 mM, respectively. Incubation mixtures for the assay of benzphetamine *N*-demethylase contained: microsomes (0.8–1.6 mg protein), 5 mM benzphetamine, 7.5 mM glucose-6-phosphate, 0.3 mM NADP, 3.0 mM nicotinamide, 100 mM Tris–HCl (pH 7.5) and 5 units of glucose-6-phosphate dehydrogenase, in a total volume of 2.0 ml. After incubating for 15 min the reaction was stopped by adding 0.7 ml of 20% trichloroacetic acid. Protein was removed by centrifugation and the supernatant solution was assayed for formaldehyde using Nash reagent (Nash, 1953).

RESULTS

Although liver weights were greater in 14-month-old rats compared with 3–5-month-old rats, there were no significant differences in recovery of microsomal membranes or in the protein content of the microsomes (Table 1). Therefore, the specific content of microsomal monooxygenase components and the specific activities of monooxygenase reactions were the same whether expressed per milligram of microsomal protein or per gram of liver. Those parameters of the microsomal monooxygenase system that were measured in both 3-month-old and 5-month-old rats (cytochrome P-450, NADPH–cytochrome P-450 reductase, benzphetamine *N*-demethylase and aniline hydroxylase) were not significantly different and values for 3-, 4-, and 5-month-old rats were pooled.

TABLE 1. LIVER WEIGHT, MICROSOMAL PROTEIN AND RECOVERY OF MICROSOMAL MEMBRANES

	Young-adult	Middle-aged
Liver weight (g)	10.9 ± 0.2	16.5 ± 0.3*
Microsomal protein† (mg/g wet weight)	29.0 ± 0.9	30.2 ± 0.7
Recovery of microsomal membranes‡ (%)	42 ± 2	43 ± 2

Male Fischer 344 rats were housed under strictly controlled environmental conditions and maintained on a semipurified diet and water *ad lib.* Young-adult rats were 3–5 months of age and averaged 270–350 g of body weight. Middle-aged rats were 14 months of age and weighed an average of 530 g. Values are means ± SE; $N \geq 6$.

*$p < 0.01$.

†Corrected for recovery of microsomal membranes.

‡The recovery of microsomal membranes was estimated by determining cytochrome c reductase activity in liver homogenate vs microsomes.

Vol. 16 1981

Aging from 5 to 14 months was accompanied by decreases in several components of the hepatic microsomal monooxygenase system, including cytochrome P-450 content (32%), NADPH–cytochrome c reductase activity (42%) and phospholipid content (41%). A less pronounced decrease (22%) in cytochrome b_5 was also seen (Table 2). Of the microsomal monooxygenase components measured, only NADPH–cytochrome P-450 reductase activity was not significantly decreased in middle-aged compared with young-adult rats.

Interestingly, liver microsomal monooxygenase activities in middle-aged rats were not uniformly decreased as a consequence of the decline in microsomal components. In fact, nitroanisole *O*-demethylation increased 44% and benzphetamine *N*-demethylation was not changed significantly. On the other hand aniline hydroxylation decreased 33% (Table 3). The substrate selectivity of these age-associated changes in microsomal drug metabolism are consistent with published observations from studies with senescent rodents (Birnbaum and Baird, 1978*a*; Birnbaum, 1980; McMartin *et al.*, 1980).

TABLE 2. COMPONENTS OF THE HEPATIC MICROSOMAL MONOOXYGENASE SYSTEM

	Young-adult	Middle-aged
Cytochrome P-450 (nmol/mg protein)	0.79 ± 0.01	0.54 ± 0.03*
Cytochrome c reductase activity† (nmol/min/mg protein)	240 ± 10	140 ± 10*
Phospholipids (nmol lipid P/mg protein)	750 ± 20	440 ± 10*
Cytochrome b_5 (nmol/mg protein)	0.46 ± 0.01	0.36 ± 0.01*
Cytochrome P-450 reductase activity‡ (nmol/min/mg protein)	0.67 ± 0.04	0.76 ± 0.08

Young-adult rats were 3–5 months and middle-aged rats were 14 months, except those used for phospholipid analyses, which were 5 and 16 months of age. Values are means ± SE; $N \geq 6$.

*$p < 0.01$.

†nmol cytochrome c reduced per min per mg microsomal protein at 23°C.

‡nmol cytochrome P-450 reduced per min per mg microsomal protein at 15°C.

TABLE 3. HEPATIC MICROSOMAL MONOOXYGENASE ACTIVITIES

	Activity (nmol/min/mg) Young-adult	Middle-aged
Nitroanisole *O*-demethylase*	1.02 ± 0.04	1.47 ± 0.11†
Aniline hydroxylase‡	0.72 ± 0.02	0.48 ± 0.03†
Benzphetamine *N*-demethylase§	4.89 ± 0.11	5.43 ± 0.32

Ages were 3–5 months and 14 months. Values are means ± SE; $N \leq 6$.
*nmol *p*-nitrophenol produced per min per mg microsomal protein at 37°C.
†$p < 0.01$.
‡nmol *p*-aminophenol produced per min per mg microsomal protein at 37°C.
§nmol HCHO produced per min per mg microsomal protein at 37°C.

The total fatty acid content was significantly less (33%) in liver microsomes from middle-aged compared with young-adult rats. Furthermore, gas chromatographic analysis of fatty acid methyl esters indicated that the composition of the microsomal fatty acids was significantly altered. Percentage increases in stearic and arachidonic acids and decreases in palmitoleic, oleic and linoleic acids were observed in microsomes from 16-month-old rats compared with those from 5-month-old rats (Table 4). Although these differences appeared small, e.g. 9.2 vs 11.6% of oleic and 28.0 vs 26.0% of arachidonic, they were similar in magnitude to the changes in microsomal fatty acid composition that occur with phenobarbital induction, e.g. 7.71 vs 10.95% of oleic and 16.39 vs 13.65% of arachidonic (Ilyas, de la Iglesia and Feuer, 1978). In spite of this significantly different distribution, aging did not alter the ratio of unsaturated to saturated or polyunsaturated to more saturated fatty acids.

TABLE 4. RELATIVE COMPOSITION OF LIVER MICROSOMAL FATTY ACIDS

Fatty acid	Weight percentage of total fatty acids Young-adult	Middle-aged
16 : 0	17.9 ± 0.2	17.0 ± 0.3
16 : 1	3.5 ± 0.1	2.6 ± 0.1*
18 : 0	20.4 ± 0.3	22.0 ± 0.4†
18 : 1	11.6 ± 0.2	9.2 ± 0.2*
18 : 2	8.3 ± 0.4	6.0 ± 0.1*
20 : 4	26.0 ± 0.4	28.0 ± 0.3*
22 : 4	4.6 ± 0.1	4.9 ± 0.3
22 : 6	2.7 ± 0.2	2.3 ± 0.3

Ages were 5 months and 16 months. Values are means for 6 rats ± SE. Total fatty acids were 377 ± 12 and 292 ± 7 μg/mg microsomal protein in young adult and middle-aged rats, respectively.
*$p < 0.01$.
†$p < 0.05$.

DISCUSSION

Previous studies of age-related changes in liver drug-metabolizing enzymes showed no significant differences between young adult and middle-aged rats (Birnbaum and Baird, 1978*b*; McMartin, 1980). However, those studies used younger animals for their middle-aged groups, as well as different strains of rats, than those used in the present study. The

marked reduction in microsomal monooxygenase components that occurred at 14 months of age in our study may indicate that age-related changes in the monooxygenase system are not related exclusively to senescence. In fact, Richardson and coworkers have observed that in male Fischer 344 rats the decline in liver microsomal cytochrome P-450 is greater between 12 and 18 months than it is between 18 and 27 months of age (Richardson, A., personal communication). Thus, it appears that in different strains of rats the specific contents of microsomal monooxygenase components decrease at different ages (or remain unchanged), and that in male Fischer 344 rats the reductions occur during the middle years.

Of interest was the finding that some drug metabolism activities were unchanged or increased in spite of substantial decreases in monooxygenase components. However, there was no decrease in the enzymatic reduction of cytochrome P-450, the putative rate-limiting step in microsomal monooxygenase reactions (Gillette and Gram, 1969; Imai, Sato and Iyanagi, 1977; Yang, Strickhart and Kicha, 1978). Although microsomes from middle-aged rats exhibited lower rates of cytochrome c reduction, this activity cannot be equated with cytochrome P-450 reduction (Vermilion and Coon, 1978; Guenthner, Kahl and Nebert, 1980). Others have shown that changes in monooxygenase activity correlate well with changes in the rate of cytochrome P-450 reduction and not very well with changes in the rate of cytochrome c reduction (Davies, Gigon and Gillette, 1969; Zannoni *et al.*, 1972; Mellon, Witiak and Feller, 1978). Our results indicated that in liver microsomes from middle-aged rats, the components that declined with age were not rate-limiting for the monooxygenation of at least two substrates.

The substrate dependency of the changes in drug metabolism activity in middle-aged rats implies that aging affected microsomal metabolism in a complex fashion that was not related to a change in the total quantity of a certain membrane component or enzyme. Cytochrome P-450, the terminal oxidase and specificity-conferring component of the monooxygenase system, exists in multiple forms. Age-related changes in the relative proportions of functionally different forms could selectively increase or decrease the metabolism of different substrates. Evidence for a selective effect of aging on different forms of cytochrome P-450 has been reported by McMartin *et al.* (1980). Using regioselective metabolism of *R*-warfarin to identify the presence of different forms of the cytochrome, they showed that liver microsomes from old Wistar rats had reduced amounts of one form of cytochrome P-450 (PB-C) and approximately equal amounts of other forms (cytochromes P-450, BNF-B and PB-B). On the other hand, Birnbaum and Baird (1978*a*, *b*) found no detectable age-related variation in rat liver microsomal cytochrome P-450 profiles, based on electrophoretograms of heme-staining polypeptides. Obviously, further work will be necessary to define clearly the effects of aging on different forms of cytochrome P-450.

Phospholipid is an integral part of the cytochrome P-450-dependent system and plays a specific role in monooxygenase reactions. Recent evidence suggests that phospholipid facilitates the formation of a binary complex between cytochrome P-450 and its reductase (Lu, Miwa and West, 1980). Reconstitution experiments using purified components of the system have demonstrated that the fatty acid moieties of the phospholipid molecule are important determinants of monooxygenase activity and that different purified forms of the cytochrome require different types and amounts of phospholipid (Strobel, Lu, Heidema and Coon, 1970; Warner and Neims, 1979). Moreover, compositional changes in the fatty acids of microsomal phospholipids are known to occur with treatments which

Vol. 16 1981

change the activity and the substrate selectivity of the microsomal enzyme system (Becker *et al.*, 1978; Ilyas *et al.*, 1978; Hammer and Wills, 1979). Thus, it seems reasonable to propose that the substrate-specific changes that occurred in the present study may have resulted also from changes in the fatty acid composition of the phospholipids.

However, other possible explanations for the substrate selectivity of the age-related changes in drug metabolism cannot be excluded. For example, age-associated changes in the composition of the microsomal membrane could differentially affect the accessibility of the monooxygenase system to the various substrates. Finally, age-related changes in the organization of microsomal components within the membrane could specifically affect monooxygenase activities.

SUMMARY

Monooxygenase components and drug metabolism activities were determined in liver microsomes from young-adult (3–5 months) and middle-aged (14 months) male Fischer 344 rats. Several components of the monooxygenase system were decreased in middle-aged rats including total cytochrome P-450, cytochrome b_5, NADPH–cytochrome c reductase activity and phospholipids. However, the reduction of cytochrome P-450 by NADPH, thought to be rate-limiting for monooxygenase activity, was not decreased. Drug metabolism activities in microsomes from middle-aged rats were increased (nitroanisole *O*-demethylation), decreased (aniline hydroxylation) or unchanged (benzphetamine *N*-demethylation). Aging decreased the microsomal content of phospholipids and changed the relative percentages of several microsomal fatty acids. The substrate selectivity of the age-related changes in drug metabolism activities may be related to changes in the fatty acid composition of microsomal phospholipids.

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Vol.
16
1981

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POST-ANOXIC RECOVERY OF MYOCARDIAL PERFORMANCE IN SENESCENT MICE

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Vol. 16 1981

INTRODUCTION

IN A WELL oxygenated heart that is receiving a normal flow of blood, free fatty acids are utilized by the heart as its main energy source. These fatty acids are used preferentially to blood glucose even in the presence of added insulin (Neely, Bowman and Morgan, 1969). However, during episodes of limited oxygen supply, a higher rate of glycolysis is maintained by the myocardium. This is in part due to the mobilization of endogenous glycogen (Brachfield and Scheuer, 1967) and an increase in the transport of blood glucose (Neely, Rovetto, Whitmer and Morgan, 1973). Animals with higher levels of myocardial glycogen display an increase in the duration of adequate myocardial performance during acute oxygen deprivation (Dawes, Mott and Shelley, 1959; Stafford and Weatherall, 1960; Scheuer and Stezoski, 1970). The maintenance of myocardial performance during anoxia may be significantly prolonged by the use of beta-blockers which increase cardiac glycogen stores (Reeves, 1963; Gelli, Enhorning, Hultman and Bergstron, 1968; Scheuer and Stezoski, 1970). A 24-h fast has also been shown to be quite effective in elevating myocardial glycogen stores (Lackey, Bunde and Harris, 1946).

Many of the above studies examined the ability of the isolated myocardium to maintain performance during anoxia. This may not always be relevant to the situation in the intact animal where respiratory (Guyton, Jones and Coleman, 1973), vascular (Guyton *et al.*, 1973), neural (Randall, 1977), and humoral (Bevegard and Shepert, 1967) factors play an important part in myocardial function. In the present study we hope to show that hepatic stores of glycogen and the ability of the body to mobilize these stores for energy are key factors in the post-anoxic recovery of myocardial performance.

MATERIALS AND METHODS

Animals

Experimental animals used in this study were male C57B1/6J mice were obtained from The Jackson Laboratories at 3 months of age. Animals were raised in our animal facility and used for study at 6 and 24 months of age. The animals were housed in their own separate rooms at a constant temperature of 24°C, 40–50% relative humidity and a 12 h light/dark cycle. They were given Purina Lab Chow *ad libitum*.

The mean life span for 115 randomly selected C57B1/6J mice was 29.7 months (median, 27 months). Average 50% survival of these male mice monitored for spontaneous deaths was 28.7 months.

Experimental design

Animals used in this study were fasted, but given water for the 24 h period prior to the beginning of the experiment. Experiments were performed on 2 young and 2 old animals during the same time daily (8–10 a.m.). Animals were anesthetized with sodium pentobarbital (80 mg/kg), the trachea surgically exposed and a cannula inserted. The thoracic cavity was exposed and positive pressure respiration was initiated using a CFP small animal ventilator (model No. 5255). The pericardial cavity was exposed and a 22 gauge needle connected to a Statham pressure transducer (P23 Dd) was inserted into the left ventricle through the apex of the heart. This pressure transducer is designed for instances where very low volume displacement characteristics (0.02 mm^3/100 mm Hg), high sensitivity (50 μV/V excitation per 100 mm Hg) and increased wave form

detail are critical. It is also designed to function within a physiological range of pressures (−50 to + 300 mm Hg). After 5 min of respiration on room air, animals were respired on 95% N_2–5% CO_2 for 3 min and then re-oxygenated with room air for an additional 5 min (recovery). At 1 min intervals during artificial ventilation selected animals were killed, hearts and livers removed and frozen in liquid nitrogen for analysis of glycogen content and phosphorylase activity. Blood samples were taken at 1 min intervals on all animals for analysis of glucose, O_2, CO_2, electrolytes and hematocrit values.

Cardiac performance

The functional ability of the heart was measured from the pressure development in the left ventricle. Information was recorded on a four channel Grass Instruments Polygraph (model No. 7D). Since it has been shown that the total energy utilization of the heart is closely related to the area beneath the systolic portion of the ventricular pressure curve, i.e. the tension-time index (TTI) (Hoffman and Buckley, 1975) the area under each pressure pulse was integrated (Grass, No. 7P10B) and the beat-to-beat heart rate was determined from the pressure pulse by triggering a tachograph circuit (Grass, No. 7B4F). Lengths of paper representing 0.5 s intervals at several known pressures were used for reference. The rate of rise and fall of the pressure pulse was determined by sending the pressure signal through an active analog differentiator (time constant = 0.05 ms).

Glycogen determination

Determination of glycogen was based on a modification of the methods of Seifter, Dayton, Novic and Muntwyler (1950) and Carrol, Longley and Roe (1956) frozen heart or liver was pulverized in a stainless steel mortar and pestle held at the temperature of liquid nitrogen. The frozen powder was digested for 30 min in 4 ml of 30% potassium hydroxide at 100°C. After digestion the tubes were cooled to 0°C in an ice-water bath and 6 ml of 95% ethanol added. Following thorough mixing, the ethanol–KOH mixture was heated at 86°C for 1 h and centrifuged at 6000 ***g*** for an additional hour. The supernatant was decanted and 1 ml of distilled water, followed by 5 ml of anthrone reagent [500 mg anthrone and 10 g thiourea in 1000 ml 72% sulfuric acid (Hassid and Abraham, 1957)] were added and mixed thoroughly. Immediately after mixing, the tubes were put into an ice-water bath to inhibit any premature color development. The tubes were then placed into a boiling water bath for 10 min and the absorbance determined at 640 nm on a Beckman spectrophotometer (model No. 25).

Phosphorylase activity

Phosphorylase (E.C. No. 2.4.1.1) activity was determined in the physiological reverse direction (dephosphorylase) by a modification of the method of Hammermeister *et al.* (Hammermeister, Yunis and Krebs, 1965) in which phosphorylase converts glucose-1-phospate (G-1-P) into free glucose and inorganic phosphorous (Pi). Frozen heart powder was placed in an incubation medium (potassium fluoride 20 mM, sodium beta-mercaptoethanol 20 mM, ethylenediaminetetracetic acid 4 mM, sodium beta-glycerophosphate 20 mM, pH 6.5) at a final concentration of 10 mg/ml (Dobson and Mayer, 1973). Total myocardial phosphorylase activity [measured in the presence of 100 mM adenosine monophosphate (AMP)] and myocardial phosphorylase A activity (measured in the absence of 100 mM AMP) were measured in this same incubation medium. Frozen liver powder was placed into two incubation mediums; total liver phosphorylase activity medium (potassium fluoride 150 mM, sodium beta-mercaptoethanol 20 mM, ethylenediaminetetracetic acid 4 mM, sodium beta-glycerophosphate 20 Mm, pH 6.5) and liver phosphorylase A activity medium [potassium fluoride 20 mM, sodium beta-mercaptoethanol 20 mM, ethylene–diaminetetracetic acid 4 mM, sodium beta-glycerophosphate 20 mM, caffeine 0.5 mM, pH 6.5 (Stalmans and Hers, 1975)] at a final concentration of 10 mg/ml. Tubes were pre-warmed to 37°C for 2 min and 80 μl of 0.465 M G-1-P were added to all tubes. Following 15 min of incubation at 37°C, 400 μl were transferred to 200 μl of 10% trichloroacetic acid (TCA). Five milliliters of distilled water were added to the tubes containing the TCA-incubation medium. Tubes were centrifuged at 6000 ***g*** for 15 min and 4 ml were removed for the determination of Pi. To the 4 ml aliquot was added 1 ml of 2.5% sodium molybdate (in 2.5 N sulfuric acid). The tubes were shaken at 1 min intervals for the next 5 min. At the end of that time 0.5 ml of 1% *p*-methylaminophenol (in 3% sodium bisulfite) were added and the tubes mixed every 10 min for 40 min. The absorbance was determined at 640 nm.

Blood glucose determination

Approximately 40 μl of blood was withdrawn from the retro-orbital sinus of animals at 1 min intervals during the 13 min respiratory period. The plasma was separated from the cells immediately, stored at −20°C and later analyzed for glucose by the glucose oxidase method (Bergmeyer and Bernt, 1965). Serum electrolytes, blood gases and hematocrit values were also analyzed at 1 min intervals during aerobic and anaerobic respiration.

Statistical analysis

Values obtained from biochemical determinations are presented as mean ± standard error of the mean. Mean values between young and old animals were compared by multifactor analysis of variance followed by multiple contrasts with the Student–Newman–Keuls test. Differences with p values ≤ 0.05 were considered significant.

RESULTS

General characteristics of myocardial performance of young and old C57B1/6J male mice during 13 min of artificial respiration are shown in Table 1. LVP profiles of old and young mice were not significantly different during the initial 5 min period of respiration with room air (Fig. 1, Table 1). During anaerobic respiration young and old animals displayed an initial transient increase in ventricular pressure followed by a decline for the remainder of anoxia. Old mice were able to re-establish pre-anoxic LVP levels during re-oxygenation while young animals underwent complete left ventricular failure. During the recovery period the 6 month old animals were not able to maintain a LVP $\geqslant$ 20 mm Hg and the heart became enlarged and flaccid.

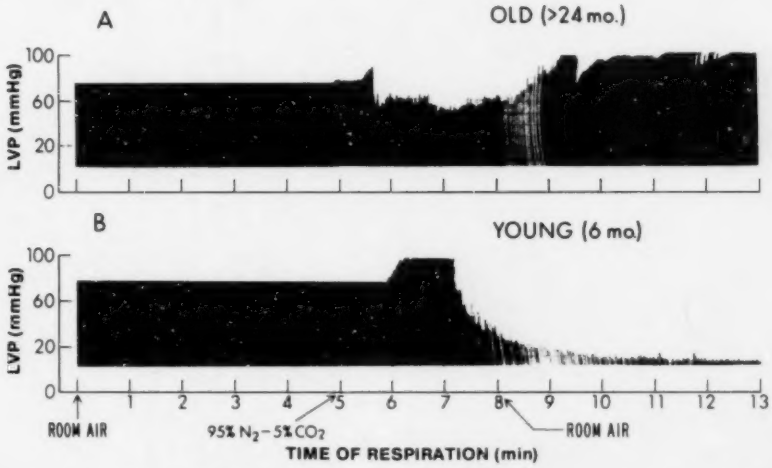

FIG. 1. Representative LVP profiles from old (A) and young (B) C57BI/6J male mice during the course of aerobic (0–5, 8–13) and anaerobic (5–8) respiration. Similar traces were obtained in an additional 24 young and 24 old mice.

Functional data of hearts from young and old animals showed similar patterns to LVP profiles of young and old animals respectively (Table 1). Although no age-related differences were noted during the 5 min period prior to anoxia, marked differences were evident during anoxia and re-oxygenation. Heart work (as shown by the tension-time index), heart rate, peak systolic pressure, and the maximum rate of pressure rise and fall, were considerably greater in old animals during the anoxic and recovery periods.

Pre- and post-fasted values of myocardial glycogen were similar in young ($0.18 \pm 0.008\%$) and old ($0.17 \pm 0.009\%$) mice. However, hepatic glycogen content in old animals ($8.4 \pm 0.56\%$) was considerably greater than that of 6 month old animals ($3.7 \pm 0.31\%$). Neither myocardial nor hepatic phosphorylase was significantly affected by a 24-h fast.

Myocardial glycogen utilization (Fig. 2a) and phosphorylase activity (Fig. 2b) were similar in young and old mice at all time points prior to re-establishment of room air. Upon renewal of room air, old hearts revealed a significant increase in glycogen stores and a concomitant decrease in phosphorylase activity. However, glycogen content and phosphorylase activity in the hearts of 6 month old mice decreased slightly throughout the recovery period.

Vol. 16 1981

TABLE 1. MYOCARDIAL PERFORMANCE OF INTACT MOUSE HEART

Time of respiration	1–5* min		6 min		7 min		8 min		9 min		10–13* min	
Age	Y ($N=25$)	O ($N=25$)	Y ($N=25$)	O ($N=25$)	Y ($N=25$)	O ($N=25$)	Y ($N=25$)	O ($N=25$)	Y ($N=25$)	O ($N=25$)	Y ($N=25$)	O ($N=25$)
Heart rate	395±30	380±25	520±42	530±38	245±23	345±24	108±22	252±22	80±20	340±18	30± 9	420±31
p	NS		NS		$\leqslant 0.05$		$\leqslant 0.05$		$\leqslant 0.05$		$\leqslant 0.05$	
LVP (mmHg)	75± 8	71± 7	90± 6	94± 5	53± 4	78± 5	30± 3	60± 4	20± 2	85± 6	10± 1	83± 6
p	NS		NS		$\leqslant 0.05$		$\leqslant 0.05$		$\leqslant 0.05$		$\leqslant 0.05$	
+dP/dTmax	163± 6	170± 5	185± 8	189± 7	120± 5	151± 4	80± 4	121± 4	58± 3	162± 5	38± 1	183± 5
p	NS		NS		$\leqslant 0.05$		$\leqslant 0.05$		$\leqslant 0.05$		$\leqslant 0.05$	
−dP/dTmax	120± 5	118± 6	142± 6	148± 4	73± 4	102± 4	41± 2	83± 3	32± 1	94± 3	23± 3	123± 5
p	NS		NS		$\leqslant 0.05$		$\leqslant 0.05$		$\leqslant 0.05$		$\leqslant 0.05$	
TTI (cm²/min)	280±11	260±10	460±12	480±12	130± 8	253± 7	43± 2	151±14	21± 1	289± 5	11± 7	335± 8
p	NS		NS		$\leqslant 0.05$		$\leqslant 0.05$		$\leqslant 0.05$		$\leqslant 0.05$	

Time of respiration = values given denote average values/min for the respiratory period indicated; Y = young (6 month) animal; O = old (24 month) animal; Heart rate = beats per min; LVP = left ventricular pressure; +dP/dTmax = maximum rate of pressure rise (mmHg/min); −dP/dTmax = maximum rate of pressure fall (mmHg/min); TTI = integrated area under the systolic portion of the left ventricular pressure curve; NS = not significant ($p \geqslant 0.1$). Values are expressed as the mean ± SEM. Number of animals are given in parentheses.

*Values did not differ significantly during the initial 5 min of respiration and the final 4 min of respiration.

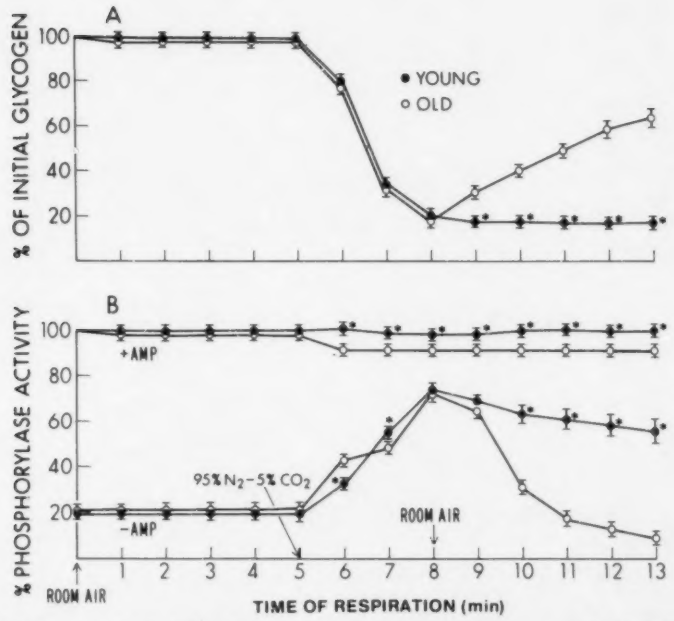

FIG. 2. Myocardial glycogen levels (A) and phosphorylase activity (B) in young and old C56B1/6J male mice during 13 min of forced ventilation. Data represents means ± SE. $^{*}p \leq 0.05$ indicates a significant difference between old and young animals under the designated testing conditions. Each data point represents average values from 25 animals sacrificed at that time of respiration. (+AMP = in the presence of adenosine monophosphate; −AMP = in the absence of adenosine monophosphate.)

A significant loss of total phosphorylase (+AMP) activity was observed in old hearts after the initiation of anaerobic respiration. This loss of total phosphorylation activity did not return to pre-anoxic levels during the recovery period (Fig. 2b).

Initial stores of hepatic glycogen in senescent animals were approx. 3-fold higher than in their younger counterparts (Fig. 3a), while pre-anoxic hepatic phosphorylase activity of young and old hearts was similar (Fig. 3b). During anaerobic respiration 24 month old animals displayed a significant increase in hepatic phosphorylase A (−AMP) activity and a slight decline in the total hepatic phosphorylase (+AMP) activity. During re-oxygenation aged animals were able to restore liver glycogen stores and phosphorylase A activity to pre-anoxic levels. Young animals showed a slight decrease in both hepatic glycogen content and phosphorylase activity during the recovery period.

Upon examination of blood glucose levels during respiration (Fig. 4), young and old mice reveal a similar time course in the rise and fall of blood sugar levels. However, the rates of rise and fall and the ultimate magnitude of plasma glucose were greater in the old animals at all time points during anaerobic respiration.

No age-related differences were noted in serum electrolytes, hematocrit, pCO_2 and pO_2 during the entire respiratory regime.

DISCUSSION

Studies utilizing the isolated working heart have revealed that increases in myocardial glycogen stores prolong anoxic survival time (Scheuer and Stezoski, 1970; Neely *et al.*,

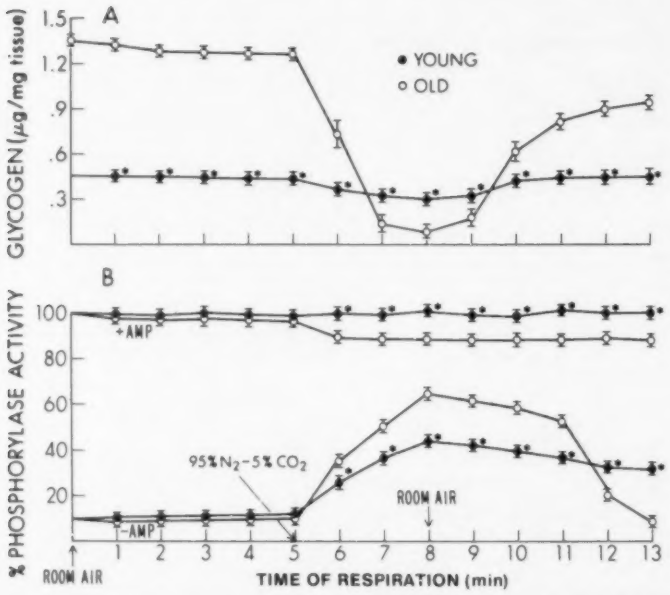

FIG. 3. Hepatic glycogen levels (A) and phosphorylase activity (B7 in young and old C57B1/6J male mice during 13 min of respiration. Data represents means ± SE. $*p \leq 0.05$ indicates a significant difference between old and young animals under the designated testing conditions. Each data point represents average values from 25 animals killed at that time of respiration. (+ AMP = in the presence of adenosine monophosphate; −AMP = in the absence of adenosine monophosphate.)

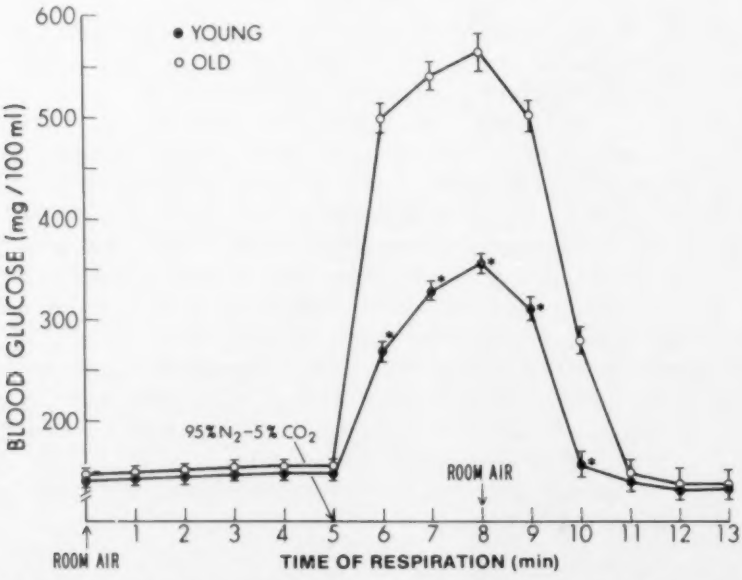

FIG. 4. Blood glucose levels during aerobic and anaerobic respiration in young and old C57B1/6J male mice. Data represents means ± SE. $*p \leq 0.05$ indicates a significant difference between old and young animals under the designated testing conditions. Each line represents average consecutive values from 25 animals sacrificed at the end of the 13 min respiratory period.

1973). Age-related increases in myocardial glycogen have been reported but in the age groups utilized in the present study no differences were encountered (Zimmerman and Capasso, 1981). However, in the isolated mouse heart utilizing a retrograde perfusion an age-related increase in the rate of glucose transport has been observed (Olafson, 1972). Although under anoxic conditions the hearts of young and old animals accelerate glucose transport to the same extent (Olafson, 1972), isolated heart studies may not reflect a true physiological workload and substrate utilization (Taegtmeyer, Hems and Krebs, 1980). Under the conditions of the present study glucose transport may be accelerated to a greater extent in the hearts of old animals (Capasso and Zimmerman, unpublished observations).

In the present study, old C57B1/6J male mice were able to maintain LVP $\geqslant$ 70 mm Hg after 3 min of anoxic respiration while young animals were unable to do so. Other myocardial performance parameters from young and old animals (Table 1) indicate that although no age-related differences exist prior to anoxia, the aged myocardium utilizes more energy during the anoxic and recovery periods. This is reflected in the aged myocardium by the maintenance of a significantly higher LVP during the anoxic and recovery periods. The maintenance of LVP by the aged myocardium may be due to age-related differences in the response of stress-related systems or metabolic abnormalities. Although the similar transient increase in LVP during anoxia to above pre-anoxic levels in both young and old animals (Table 1, Fig. 1) may reflect the positive inotropic effect of catecholamines, additional studies have shown that the aged myocardium has depressed contractility (Alpert, Gale and Taylor, 1967), prolonged contraction duration (Lakatta, Gerstenblith and Angell, 1975), and decreased drug responsiveness (Lakatta *et al.*, 1975*a*). All these factors are associated with a decreased ability to withstand an acute stress (Guyton *et al.*, 1973; Lakatta *et al.*, 1975*a*). Therefore, other factor(s) may be aiding the aged myocardium in its response to the anoxic stress.

Vol. 16 1981

Metabolically, since glycogen is the major endogenous substrate utilized by the heart during anoxia (Brachfeld and Scheuer, 1967), its amount and rate of degradation play an important part in myocardial survival during and subsequent to anoxia (Brachfeld and Scheuer, 1967; Hearse and Chain, 1972; Scheuer and Stezoski, 1970; Rovetto, Whitmer and Neely, 1973). The key enzyme involved in the mobilization of myocardial glycogen during anoxia is phosphorylase (Dobson and Mayer, 1973) and no age-related differences in the activation of phosphorylase appear to be present (Fig. 2). However, the total phosphorylase present in the aged myocardium declines significantly during anoxia (Fig. 2) (Zimmerman and Capasso, 1981). This is probably the result of phosphorylase molecules that are less stable and denature under the rigorous conditions present in an anoxic cell (Capasso and Zimmerman, 1980). Since myocardial glycogen stores and utilization (Fig. 2a and b) are similar in young and old animals it would appear that there are other energy-producing factor(s) responsible for the age-related differences in the maintenance of myocardial performance by the aged mouse.

Hepatic glycogen represents an additional source of stress-related energy. After a 24 h fast liver glycogen in young and old animals has decreased but the aged animal still maintains a 3-fold higher concentration of liver glycogen than its younger counterpart. With the onset of anoxia aged animals not only have considerably more liver glycogen but they are able to mobilize these stores at a quicker rate (Fig. 3*a*). This is reflected in the significantly greater activation of liver phosphorylase by the aged animal (Fig. 3b). Similar to the aged myocardium, hepatic levels of total phosphorylase decline during

anoxia, probably as the result of a less stable molecule (Capasso and Zimmerman, 1980). The mobilization of hepatic stores of glycogen during anoxia is reflected by the change in serum levels of glucose (Fig. 4). Young and old glucose levels in the blood rise significantly during anoxia but the old animal is able to maintain higher blood sugar levels at all times during anoxia. In order to fully mobilize these glycogen stores, hepatic phosphorylase is activated to a greater extent in the aged animal (Fig. 3a).

We believe that increased levels of liver glycogen in the old animal coupled with the aged myocardium's ability to transport glucose at an increased rate (Olafson, 1972; Capasso and Zimmerman, unpublished observations) are significant factors in the maintenance of left ventricular performance during and after anoxic respiration. During situations where oxygen supply to the heart is limited, the survival of the heart will depend on endogenous stores of glycogen and ability to maintain and transport adequate amounts of glucose from the blood to the heart. If blood glucose levels can be kept at a level necessary to maintain a higher transport rate, the myocardium may be able to prolong performance during anoxia.

SUMMARY

In studies with intact open-chested male mice, the ability of the myocardium to maintain left ventricular pressure (LVP) during and subsequent to 3 min of anaerobic respiration has been studied. The hearts of young (6 months) and old (24 months) animals revealed dissimilar pressure profiles throughout the anoxic and recovery periods. Upon re-establishment of aerobic respiration, only old animals were able to restore LVP to pre-anoxic levels. Indices of external myocardial work were similar in young and old hearts prior to anoxia and declined significantly more in young animals during anoxia and recovery.

In order to localize the energy source for the recovery of myocardial performance by old animals myocardial and hepatic levels of glycogen and phosphorylase were assessed. Cardiac glycogen and phosphorylase levels were similar in both young and old animals prior to the recovery period. Upon recovery from anoxia, old animals were able to restore myocardial glycogen content and phosphorylase activity towards pre-anoxic levels while the hearts of young animals failed to do so. Pre-anoxic hepatic glycogen content in old animals was significantly greater than young animals while phosphorylase levels were similar in both groups prior to anaerobic respiration. Hepatic glycogen utilization and phosphorylase activation throughout the respiratory period were similar to that seen in the myocardium from both young and old animals with old animals displaying a greater utilization of glycogen and a greater activation of phosphorylase during anoxia and a greater tendency to return to pre-anoxic levels during the recovery period. In addition, blood glucose levels during anoxia were significantly lower in young animals when compared to values obtained from old animals. Under these conditions, increases in liver glycogen may be responsible for the maintenance of myocardial performance during and after anoxia by old mice.

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Vol.
16
1981

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A PRACTICAL PRESERVATION METHOD FOR HUMAN DIPLOID FIBROBLASTS IN AGING RESEARCH

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Vol.
16
1981

INTRODUCTION

HUMAN diploid fibroblasts have a finite lifespan in culture (Hayflick and Moorhead, 1961; Jacobs, Jones and Baille, 1970; Nichols *et al.*, 1976). For this reason, they have been widely used as a model system for the study of cellular aging *in vitro* (Cristofalo, 1972). Recently, a new human diploid cell strain (TIG-1) was established from a female fetal lung by the project team for research on cellular aging in the Tokyo Metropolitan Institute of Gerontology (Ohashi *et al.*, 1980). When normal fibroblasts are used for aging research, it is always necessary to know the population-doubling number of these cells, and to maintain the original properties for a period of preservation in frozen state.

In 1949 Polge, Smith and Parker found that fowl spermatozoa frozen in 1.1 M glycerol recovered with little damage after thawing. In the presence of glycerol cells of many different types have been protected against damage during freezing, and have been successfully grown after preservation by freezing, and have been successfully grown after preservation by freezing for a long period (Smith, 1952; Levelock, 1953; Scherer and Hoogasian, 1954; Swim, Haff and Parker, 1958). Subsequently, it has been reported that dimethyl sulfoxide (DMSO) also acts as a protective substance against damage of human erythrocytes by freezing (Levelock and Bishop, 1959), and that, moreover, survival of primary chick embryo fibroblasts when frozen, is much better with DMSO than with glycerol (Dougherty, 1962). Long-term preservation of cells and tissues has been reviewed (Meryman, 1966; Mazur, 1970; Pegg, 1976; Ashwood-Smith, 1980), but that of human diploid fibroblasts with glycerol has been very little reported (Hayflick and Moorhead, 1961). Moreover, toxicity of DMSO for HeLa cells has been reported (Malinin and Perry, 1967). It has also been recently shown that DMSO induces cell differentiation of many cultured mammalian cells (Friend, Scher, Holland and Sato, 1971; Kimhi, Palfrey, Spector, Barak and Littauer, 1976; Klung *et al.*, 1976; Miranda, Nettle, Khan, Brockbank and Schonberg, 1978) and increases the activities of lysosomal enzymes in human skin fibroblasts (Scanlin and Glick, 1978).

Our purpose in this paper, therefore, is to study a practical preservation method for human diploid fibroblasts by using glycerol as an additive, avoiding the possibility of differentiation induced by DMSO. This study was also started because quantitative recovery of frozen cells was unsatisfactory when the cells were frozen using glycerol according to the method of Hayflick (Hayflick and Moorhead, 1961; Hayflick, personal communication). A practical method for the preservation of human diploid fibroblasts which permits the recovery of a high proportion of the cells frozen with glycerol was developed.

MATERIAL AND METHODS

Cell culture

Human diploid fetal lung fibroblasts (TIG-1 cells) were maintained in culture as described previously (Ohashi *et al.*, 1980). The cells were cultured in plastic dishes (Falcon, 3002) containing 5 ml of Eagle's basal medium (BME) supplemented with 10% fetal bovine serum (FBS, Gibco, lot No. R 472320), 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C under humidified 5% CO_2–95% air. The cells were removed from the dish by treatment with 0.25% trypsin (Difco, 1 : 250) in Ca^{2+}- and Mg^{2+}-free phosphate buffer (PBS) at 37°C for 15 min, and subcultivated with 1 : 4 split ratio every 1 week. The cells were judged mycoplasma-free by the method of Kihara, Ishida and Okumura (1981). The cells at 19–25 population doublings (PD) were used for the freezing experiments.

Preservation of cells by freezing

Conventional method. The conventional method of preservation of cells by freezing is in accordance with the method of Hayflick (Hayflick and Moorhead, 1961; Hayflick, personal communication). The cells were harvested by trypsinization from 10 dishes at 7 days (at the confluency) after subcultivation. The cells were suspended in 40 ml of BME supplemented with 10% FBS and antibiotics (growth medium) and centrifuged at 170 ***g*** for 5 min. The cells were resuspended in 10 ml of BME supplemented with 10% FBS and 10% glycerol (conventional freezing medium). Glycerol (Merk) for fluoresence microscopy was used. One part of the cell suspension was used for counting initial inoculum cell number by a Coulter counter. Each 1 ml of cell suspension containing usually approx. 4×10^6 cells/ml (corresponding to cell number in one dish) was dispensed into a 1.2 ml glass ampoule (Wheaton Sci.). The ampoules were then sealed and placed in the refrigerator at 4°C for 3 h. The cells in the ampoule after agitation were frozen to −35°C at a rate of 1°C/min by using a Slow Freezing System (Nippon Use Sci., Model CS-201) which was equipped with a program control system to set the rate of temperature decrease. The frozen cells were stored in liquid nitrogen for 1 week to 1 month. When the frozen cells were reconstituted, the ampoule was removed from the liquid nitrogen and immediately plunged into a 40°C water bath until the contents were thawed. After the ampoule was placed in a 37°C water bath for 15 min, the cell suspension was diluted by the addition of 9 ml of the growth medium. The cells were centrifuged at 170 ***g*** for 5 min, and the medium with glycerol was removed. The cells were resuspended in 5 ml of the fresh growth medium. An aliquot (0.4 ml) of the material was taken for the determination of viability. The remainder was plated in the dish, and incubated for 16 h at 37°C in a humidified CO_2 incubator. The dish was washed twice with 2.5 ml of PBS. Cells attached to the dish were suspended with 5 ml of the growth medium after trypsinization, and counted by the Coulter counter.

Recommended method. The recommended method was obtained by the modification of the conventional method. Cells/ampoule $1–10 \times 10^6$ were frozen in the medium with 30% FBS and 15% glycerol to −35°C at a rate of 1°C/min after pretreating at 4°C for 3 h. The ampoule was stored in liquid nitrogen. The ampoule that was to be reconstituted was removed from the liquid nitrogen, and the contents were rapidly thawed by shaking in a 40°C water bath. After standing for 15 min in a 37°C water bath, the ampoule was cut, and the cells (1 ml) were diluted by the addition of 4 ml of the growth medium. The cells were plated in the dish, and cultured at 37°C in the humidified CO_2 incubator.

Measurement of viability and cell attachment

Viability test of cells was done by staining with 0.2% nigrosin in 0.9% NaCl solution. Unstained viable cells and stained dead cells were counted in a hemocytometer within 3–15 min after staining. Viability was calculated by dividing the number of unstained viable cells by the number of total cells. The number of initial inoculum cells and the number of cells attached to the dish after 16 h incubation were counted by the Model ZBI Coulter counter. The percentage of cell attachment was calculated by dividing the number of attached cells by the initial cell number. This calculation gives an index of the recovery of cells which are able to grow into subsequent generations attaching to a substratum.

RESULTS

Effects of freezing conditions on viability and cell attachment

Preservation of cells by freezing was mostly affected by freezing conditions (freezing medium and freezing rate), thawing conditions (temperature at thawing and thawing rate), and cellular conditions (cell density and growth conditions). At first, the effects of various concentrations of glycerol added as an additive in freezing medium were examined. The results summarized in Fig. 1 show that in control (conventional method using the medium with 10% FBS and 10% glycerol), the viability was 82.5%, and the cell attachment (recovery) was 32.9%, indicating that approx. 40% of viable cells was able to

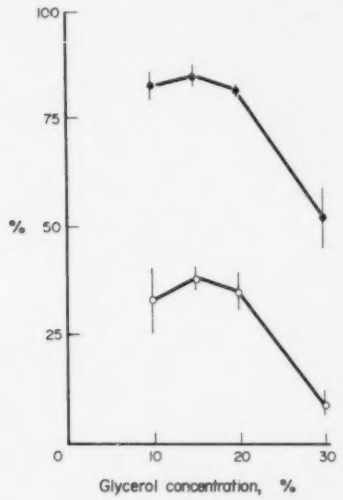

FIG. 1. Effects of glycerol concentrations in the freezing medium on viability and cell attachment. Approximate 4×10^6 cells/ampoule were frozen in the medium (BME) containing 10% FBS and different concentrations of glycerol. Frozen cells were thawed by standing in a 40°C water bath. After thawing cells were plated in the dish according to the conventional method in Material and Methods. Viability and per cent of cell attachment were determined according to the method in Material and Methods. Each value represents the mean ± S.D. of at least four determinations. ●, viability; ○, cell attachment.

attach to the dish. The optimum concentration of glycerol for both viability and cell attachment was 15% although there was not significant difference between 10 and 20% glycerol. The concentration of 30% glycerol was toxic for the survival of frozen cells. The toxic effects of high concentrations may be due to be manifestations of osmotic shock during the removal of the additive.

Secondly, the conditions of the freezing medium containing various concentrations of FBS were examined. The results summarized in Fig. 2 show that the concentration of FBS was important to the recovery of cells frozen with glycerol. The cell attachment was highest (1.5-fold of control) when cells were frozen in freezing medium with 30% FBS. However, higher concentrations (50–90%) of FBS had almost no effect on cell attachment. This effect of FBS in high concentrations may be due to some inhibitors on cell attachment. The viability was scarcely affected by serum concentrations though slightly higher with 30% FBS.

In both the viability and the attachment of cells, pH in freezing medium with glycerol was a critical factor as shown in Fig. 3. Both parameters increased linearly between pH 6.8 and 7.8, achieved optimum condition at pH 7.8, and decreased slightly at pH 8.2.

Stulberg, Soule and Berman (1970) have reported that the rate of freezing is a critical factor for the survival of cells frozen in the glycerol medium, and that the survival of cells is best when the temperature is lowered at a rate of 1°C/min throughout the range of 4°–−25°C, then rapidly to below −50°C. The effect of the freezing rate on the survival of frozen cells was examined at a rate of 0.5, 1 and 2°C/min. The recovery of cells frozen at a rate of 0.5 and 2°C/min, 28.3 and 20.2%, respectively, was worse than (32.9%) at a rate of 1°C/min, although the viability was not different in these rates. Cells frozen directly in liquid nitrogen suffered severe damage, and almost all (97%) the cells failed to attach to the dish.

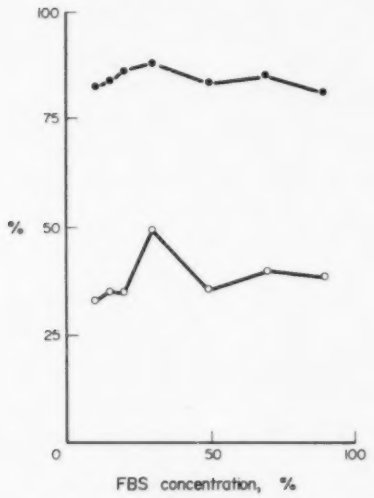

FIG. 2. Effects of FBS concentrations in the freezing medium on viability and cell attachment. Cells (*ca.* 4×10^6 cells/ampoule) were frozen in the medium containing different concentrations of FBS and 10% glycerol and thawed according to the conventional method. Each value represents the mean of duplicate determinations, except for that of 10% FBS (control) obtained from 38 determinations. Symbols as in Fig. 1.

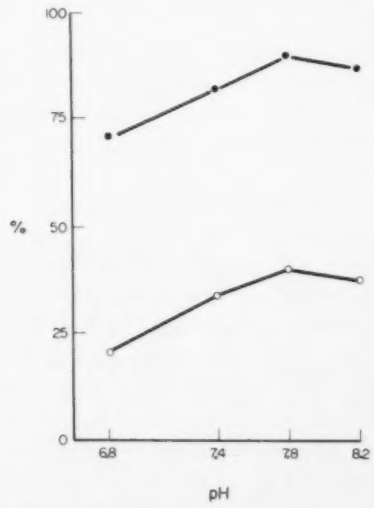

FIG. 3. Effect of pH in the freezing medium on viability and cell attachment. Cells (*ca.* 4×10^6 cells/ampoule) were frozen in the medium containing 10% FBS, 10% glycerol and 25 mM Hepes buffer adjusted to each pH (6.8, 7.4, 7.8 and 8.2) and thawed according to the conventional method. Each value represents the mean of duplicate determinations. Symbols as in Fig. 1.

Effects of thawing conditions on viability and cell attachment

Human diploid fibroblasts frozen in the conventional freezing medium were thawed under different conditions. The results (Table 1) show that viability and cell attachment were improved, 1.07 and 1.27 times respectively, by rapidly thawing at the rate of 6.5°C/s shaking the ampoule in a 40°C water bath compared to thawing at the rate of 3.1°C/s

TABLE 1. EFFECTS OF THE TEMPERATURE AND RATE OF THAWING ON VIABILITY AND CELL ATTACHMENT

Condition (Exp. No.)	Thawing rate	Viability (%) Mean ± S.D.	Cell attachment (%) Mean ± S.D.
Before freezing		97.9 ± 0.93	100
40°C, shaking (2)	6.5°C/s	88.3 ± 1.90	41.7 ± 3.50
40°C, standing (38)	3.1°C/s	82.5 ± 3.48	32.9 ± 7.71
25°C, standing (2)	—	52.6 ± 9.05	13.6 ± 2.35
4°C, standing (2)	0.6°C/s	47.1 ± 4.30	7.34 ± 1.43

Cells (4×10^6 cells/ampoule) frozen in the medium with 10% FBS and 10% glycerol were thawed at the temperature given in this table.

standing the ampoule in the 40°C water bath (conventional method), and that cell damage was markedly induced when the cells were thawed more slowly at 25°C and lower temperatures.

Next, we examined a standing period after thawing to allow repair of cell damage induced by the freeze–thawing procedure. Table 2 shows that no difference was found in the viability under these conditions so far examined, while the cell attachment slightly increased when cells were diluted by the addition of the growth medium at 15 min after thawing, and centrifuged 15 min later. In any case, time to repair cell damage seems not to be a critical factor for the survival of frozen cells.

TABLE 2. EFFECTS OF STANDING PERIOD AFTER THAWING ON VIABILITY AND CELL ATTACHMENT

Condition	Viability (%) Mean ± S.D.	Cell attachment (%) Mean ± S.D.
Before freezing	97.9 ± 0.93	100
0–0* (2)	85.6 ± 0.22	31.8 ± 8.30
15–0 (38)	82.5 ± 3.48	32.9 ± 7.71
0–15 (2)	85.2 ± 0.85	31.3 ± 6.00
15–15 (2)	84.7 ± 1.45	36.5 ± 0.80
30–0 (2)	81.8 ± 4.85	30.8 ± 4.75

*0–0: Diluted at 0 min after thawing, and centrifuged at 0 min after dilution.

Cells (4×10^6 cells/ampoule) were frozen and thawed according to the conventional method. After thawing cells were diluted by the addition of 9 ml of the growth medium at 0, 15, or 30 min and centrifuged at 0 or 15 min after dilution.

Effects of cellular conditions on viability and cell attachment

As shown in Table 3, the viability decreased 0.88-fold, but the cell attachment increased 1.43-fold in rapidly growing cells compared to cells at confluent culture. In cultures where we had changed the medium on the day before, the cell attachment increased slightly (1.11-fold of control) although the viability was not different. These results seem to be related with the findings that were obtained in synchronized Chinese

Vol. 16 1981

TABLE 3. EFFECTS OF CELLULAR CONDITIONS ON VIABILITY AND CELL ATTACHMENT

Condition (Exp. No.)	Viability (%) Mean ± S.D.	Cell attachment (%) Mean ± S.D.
Before freezing	97.9 ± 0.93	100
Confluency		
Without medium change (38)	82.5 ± 3.48	32.9 ± 7.71
With medium change (4)	81.4 ± 2.09	36.5 ± 3.66
Before freezing	97.3	100
Rapidly growing		
Without medium change (2)	72.4 ± 0.55	47.2 ± 0.55

Cells (4×10^6 cells/ampoule) which reached at confluency (7 days after subcultivation) with or without medium change in the day before and which were rapidly growing (2 days after subcultivation) were frozen and thawed according to the conventional method in Material and Methods.

hamster cells frozen in the medium with 15% glycerol (Koch, Kruuv and Bruck-Schwaiger, 1970) and that were obtained in HeLa cells in 10% DMSO medium (Terasima and Yasukawa, 1977). Rapidly growing cells were not used in this paper, because these include cells at different phases of cell cycle, and some of these cells may be able to double during 16 h incubation.

The results summarized in Fig. 4 indicate that the cell density must be adjusted at least to 1×10^6 cells/ml when cells were frozen in 10% glycerol medium. As the cell density at 5×10^5 cells/ml gave unsatisfactory results, the cell density must be adjusted once more to the proper amount of the freezing medium after centrifugation.

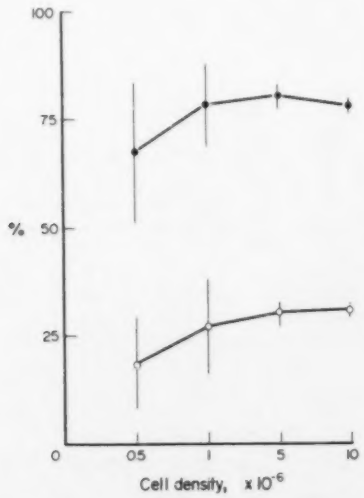

FIG. 4. Effects of the cell densities in the frozen state on viability and cell attachment. Each density of cells was frozen and thawed according to the conventional method. Each value represents the mean ± S.D. of at least four determinations. Symbols as in Fig. 1.

Effects of the serum addition and dilution on the repair of cell damage due to the freeze–thawing procedure

As described above, 60% of viable cells has failed to attach to the dish when human diploid fibroblasts were frozen by the conventional method. This result suggests that in addition to cell loss, cell damage to an extent at which cells were incapable of attaching to the dish, though capable of excluding the nigrosin dye, had been induced by centrifugation. The serum has been reported to promote cell attachment to the substrate in culture cells (Fisher, Punk and Sato, 1958; Taylor, 1961). The effects of the serum on the attachment of viable, unattached cells to the dish were examined. When 50% FBS at a final concentration was added to the growth medium after thawing, the cell attachment significantly increased as observed in Fig. 5, and the result was a maximum value (53.6% attachment) obtained from the centrifugation method. The addition of more than 70% FBS reduced the cell attachment. On the other hand, more serum added to the medium resulted in higher yields of viable cells. However, the judgement of viability by nigrosin exclusion was difficult in the presence of high concentration of serum, because the permeability of biomembrane might be affected by the high concentration of macromolecules in the medium. Consequently, the viability may be shown to be higher.

Vol. 16 1981

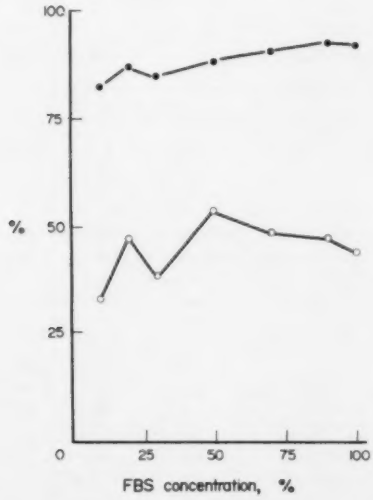

FIG. 5. Effects of FBS concentrations added to the growth medium after reconstitution on cell damage caused by freezing and thawing. Cells (*ca.* 4×10^6 cells/ampoule) were frozen and thawed according to the conventional method. After centrifugation FBS was added to the growth medium. Each value represents the mean of duplicate determinations, except for that of control (10% FBS) obtained from 38 determinations. Symbols as in Fig. 1.

Next, cells (1 ml) thawed according to the conventional method were diluted by the addition of 4, 9, or 29 ml of the growth medium, and plated in the dish without centrifugation (dilution method). The results in Table 4 show that the cell attachment was much better in the dilution method, and, in fact, it was the maximum value (61.9%) obtained from the 10% glycerol medium, as cells were diluted 5-fold with the medium.

TABLE 4. EFFECT OF DILUTION ON THE RECOVERY OF CELL DAMAGE CAUSED BY FREEZING AND THAWING

Condition (Exp. No.)		Viability (%) Mean ± S.D.	Cell attachment (%) Mean ± S.D.
Before freezing		97.9 ± 0.93	100
Centrifugation	(38)	82.5 ± 3.48	32.9 ± 7.71
Dilution, 5-fold	(14)	85.7 ± 2.83	61.9 ± 7.30
Dilution, 10-fold	(2)	83.5 ± 2.05	50.7 ± 0.65
Dilution, 30-fold	(2)*	81.5 ± 2.30	41.1 ± 1.50

*Flask (Falcon, 3012) was used.

Cells (4×10^6 cells/ampoule) reconstituted according to the conventional method were diluted by the addition of 4, 9, or 29 ml of the growth medium and plated in the dish without centrifugation (dilution method), contrary to the control with centrifugation.

The reason that the recovery of cells frozen in the 10% glycerol medium remarkably increased 5-fold dilution may be due to reduce osmotic shock during the removal of the additive as compared with 10- and 30-fold dilution. However, the viability was not different.

The effect of final concentrations of glycerol on cell attachment was further examined. It is clear from Table 5 that the presence of less than 5% glycerol at a final concentration is not inhibitory on cell attachment in human diploid fibroblasts like in chick embryo fibroblasts reported previously (Porterfield and Ashwood-Smith, 1962).

TABLE 5. CELL ATTACHMENT IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF GLYCEROL

Glycerol conc. (%)	FBS conc. (%)	Cell attachment (%)	
0	10	93.2	(100)
1	10	92.9	(99.8)
2	10	90.9	(97.6)
3	10	92.6	(99.5)
5	10	90.3	(97.0)
10	10	77.4	(79.9)
0	14	94.4	(101.4)
3	14	91.6	(98.4)
5	14	90.1	(96.8)
10	14	78.0	(83.8)

Cells harvested at confluency (7 days after subcultivation) were suspended in the growth medium with or without glycerol. Each dish contained 1.25×10^6 cells and the concentration of glycerol given in this table was incubated for 16 h in the humidified CO_2 incubator. Cells attached to the dish were counted by the Coulter counter after trypsinization. Each value represents the mean of duplicate determinations.

Recommended method obtained by modification of the conventional method

Finally, we concluded that the conditions increased cell attachment and/or viability were as follows: (1) The concentration of FBS in the freezing medium is 30%, (2) the glycerol concentration is 15%, (3) the pH of the freezing medium is 7.8, (4) the freezing rate is 1°C/min, (5) the contents in the ampoule are thawed rapidly, but not bubbled, by agitation in a 40°C water bath, (6) removal of effects of the additive is carried out by diluting 10-fold with the growth medium at 15 min after thawing and then by centrifuging after 15 min (centrifugation method), or by diluting 5-fold with the growth medium at 15 min after thawing without centrifugation (dilution method), (7) the cell density is adjusted to 1–10 $\times$ 10^6 cells/ampoule, (8) the concentration of FBS added to the medium after reconstitution is 50%. The best result obtained by the combination of these optimal conditions [except for addition of 25 mM Hepes buffer (pH 7.8) to the freezing medium and addition of 50% FBS to the medium after thawing] showed 90.8% viability and 78.8% recovery (Table 6). Consequently, in this modified method (recommended method) the proportion of attached cells to viable cells was 87% and this value is 2-fold as contrasted with the value (40%) obtained by using the conventional method. Neither the slow dilution method nor the step-wise dilution method that were used to reduce osmotic damage associated with the removal of additive after thawing gave higher survival than the recommended method (directly 5-fold dilution) (Table 6). Under the conditions combined above (1)–(8), both the viability and the recovery were reduced compared with the best conditions described above. In an experiment where cells were frozen by using 2 ml polypropyrene tubes (Nunclon) instead of glass ampoules under the best conditions, both the viability and the recovery were reduced significantly as shown in Table 6.

A protective effect of DMSO on freezing damage was compared with that of glycerol by using the centrifugation method, for removal of DMSO used as an additive. Both the survival and the recovery of cells frozen with DMSO were almost the same as that with glycerol in the modified method. In the modified method with glycerol the recovery was 53.5% (1.63-fold of control) as observed in Table 6. Therefore, changes in population doubling number through the preservation procedures can be avoided even by the centrifugation method if cells harvested from 2 dishes are frozen within an ampoule by the modified method.

The growth and the lifespan of human diploid fibroblasts frozen

The method (cell attachment) used for measuring the survival ratio in the experiments described above does not give any indication of growth ability of cells attached to the dish. The growth of frozen cells was then compared with that of unfrozen cells as control (Fig. 6). In contrast to 93.8% attachment of the control cells (98.9% viability), 69.6% of the frozen cells (91.4% viability) were able to attach to the dish after 16 h incubation. The rate of subsequent multiplication was not significantly different between the frozen cells and control cells. Therefore, cells attached to the dish after reconstitution seem to suffer little or no impairment of their ability to grow.

The cells frozen at PD 25 by the recommended method were subcultivated with split ratio 1 : 4 during the lifespan after thawing (Fig. 7). The cell density on the 7th day after subcultivation gradually declined with the progress of population doublings. The cell density and the finite lifespan (67 population doublings) were not different between the frozen cell culture and the control cell culture. Therefore, the recommended method is useful for the storage of human diploid fibroblasts.

Vol. 16 1981

TABLE 6. COMPARISON BETWEEN THE CONVENTIONAL AND MODIFIED METHODS, AND THAT BETWEEN GLYCEROL AND DMSO

Condition (Exp. No.)		Viability (%) Mean ± S.D.	Cell attachment (%) Mean ± S.D.
Before freezing		97.9 ± 0.93	100
Centrifugation			
Glycerol			
BME-10%FBS-10%Gly	(38)*	82.5 ± 3.48	32.9 ± 7.71
BME-30%FBS-15%Gly	(6)†	88.9 ± 1.96	53.5 ± 3.92
DMSO			
BME-10%FBS-10%DMSO	(4)‡	85.9 ± 3.50	52.9 ± 8.42
BME-30%FBS-15%DMSO	(2)‡	82.0 ± 0.20	53.3 ± 2.45
Dilution			
5-fold dilution			
BME-10%FBS-10%Gly	(14)*	85.7 ± 2.83	61.9 ± 7.30
BME-30%FBS-15%Gly	(8)§	90.8 ± 1.53	78.8 ± 3.12
BME-30%FBS-15%Gly-Hep	(4)‖	90.4 ± 1.31	67.9 ± 5.16
BME-30%FBS-15%Gly-Nunc	(4)¶	80.9 ± 0.51	63.7 ± 2.07
Step-wise dilution			
BME-30%FBS-15%Gly	(4)**	89.5 ± 2.13	78.8 ± 4.26
Slow dilution			
BME-30%FBS-15%Gly	(4)††	90.3 ± 0.65	79.7 ± 2.14

*Cells were frozen in the medium with i0% FBS and 10% glycerol and thawed by standing in a 40°C water bath. After thawing cells were plated in the dish after centrifugation according to the conventional method in Material and Methods or only dilution without centrifugation (dilution method).

†Cells were frozen in the medium with 30% FBS and 15% glycerol and thawed rapidly by shaking in a 40°C water bath. After thawing cells were plated in the dish after centrifugation (modified method).

‡Cells were frozen and thawed according to the method of * or †, except for using DMSO instead of glycerol.

§Cells were frozen and thawed according to the modified method of †, except for using dilution method instead of centrifugation method (recommended method).

‖Cells were frozen and thawed according to the method obtained from the combination of all optimums of (10)–(8) (see Results).

¶Cells were frozen and thawed according to the recommended method, except for using Nunc polypropyrene tubes instead of Wheaton glass ampoules.

**Cells frozen in the medium with 30% FBS and 15% glycerol were thawed and diluted according to the method of Mazur *et al.* (1969).

††Cells frozen in the medium with 30% FBS and 15% glycerol were thawed and diluted according to the method of Evans *et al.* (1962).

DISCUSSION

The relationship between viability and cell attachment is summarized in Fig. 8. In the centrifugation method, the viability was mostly the range of 80–90%, but the cell attachment was significantly different (21–48%) even when the viability showed almost the same value. The results indicate that it is questionable that the judgement of survivals (or recovery) of cells frozen can be made with dye exclusion as the only viability test, although viability is a standard of survivals at 70% or less. Therefore, it is necessary to measure not only viability but cell attachment in the preservation of human diploid fibroblasts by freezing.

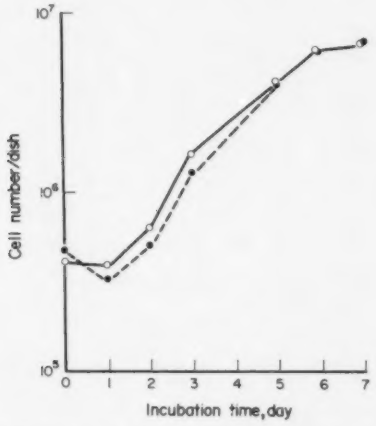

FIG. 6. Growth of frozen and unfrozen cells. Cells (4.6×10^6 cells/ampoule) were frozen in the medium with 30% FBS and 15% glycerol. On the following day the frozen cells were rapidly thawed by shaking in a 40°C water bath, and plated in 10 dishes at 4.60×10^5 cells/dish. On the other hand, unfrozen cells were plated in 10 dishes at 4.06×10^5 cells/dish. The same medium was used for both groups. Two dishes were removed each day from each group and the cells were counted by the Coulter counter. The medium was changed at 1, 3 and 5 days. ○, control (unfrozen) cells; ●, cells frozen with 15% glycerol.

Vol. 16 1981

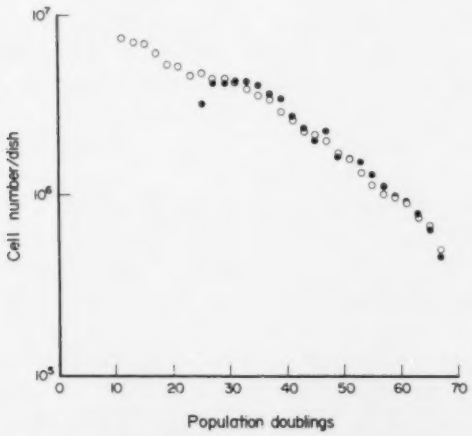

FIG. 7. Changes in cell density of frozen and unfrozen cells during the lifespan. Cells were frozen and thawed according to the recommended method. The cells were subcultivated with split ratio 1 : 4 once a week, and the cell number was counted by a hemocytometer. ○, control (unfrozen) cells; ●, cells frozen by the recommended method.

Not all cells attached to a substrate are able to grow, as is shown by the behaviour of irradiated cells. We were afraid that some of the cells attached to the dish after reconstitution might have lost the ability of growth through freezing and thawing damage. Fortunately, no difference between the growth of control (unfrozen) cells and that of cells frozen with glycerol was observed (Fig. 6). It has already been reported that primary chick embryo fibroblasts frozen with 10% DMSO suffered little or no impairment in their ability to grow (Dougherty, 1962). Consequently, cell attachment can be used to judge the

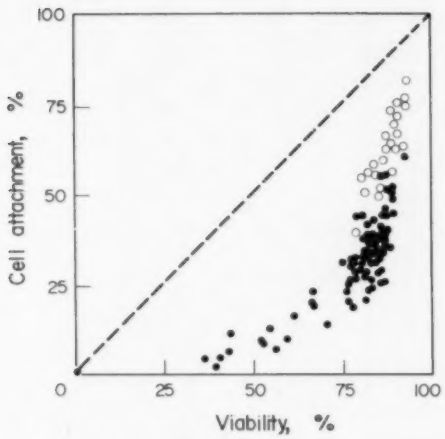

FIG. 8. Relationship between viability and cell attachment. Each point represents the value obtained from this experiment. ●, centrifugation method; ○, dilution method.

survival ratio of frozen human diploid fibroblasts. Plating efficiency was not used for the measurement of the survival ratio of frozen cells in this experiment as even unfrozen human diploid fibroblasts have indicated low plating efficiency (Nichols *et al.*, 1976).

It is presumed that cell loss and cell damage were induced by centrifugation to remove glycerol or DMSO additive. In this experiment, it was found that viability was not different between the centrifugation and the dilution methods, but cell attachment increased by approx. 30% in the dilution method (5-fold dilution) in contrast to the centrifugation method (Table 4). In addition, 15% of total cells were lost by centrifuging at 170 ***g*** for 5 min without freezing (unshown data). Accordingly, the cell loss by centrifugation is calculated to be only 4–5% out of the attached cells (30%), judging from increase by the dilution method. The remainder (25%), i.e. most of the difference of cell attachment between the centrifugation and the dilution methods, seems to be due to cell damage which is undetectable by the dye exclusion test.

It has been reported that serum is important for the survival of frozen cells (Dougherty, 1962), and it can increase cell attachment in several different cell types (Fisher *et al.*, 1958; Taylor, 1961; Culp and Buniel, 1976; Grinnel, 1976; Feldman and Wong, 1977). In TIG-1 cells, serum is important for cell attachment, and the results showed that 93–97% of unfrozen cells could attach to the dish in the medium with 10% FBS in contrast to only 33% in the medium without serum (unpublished data). FBS was further added to the medium for the purpose of recovering the ability of attachment of viable, unattached cells after reconstitution. As a result, 50% FBS added to the culture medium increased remarkably the attachment of cells reconstituted with the conventional method (Fig. 5). About half of viable, unattached cells could recover the ability of attachment by the addition of 50% FBS, perhaps by specific serum components adsorbed to the substratum surface (Grinnell, 1976, Heckman, Vroman and Pitlick, 1977) and/or cell surface. Because the cell attachment increased markedly when TIG-1 cells were frozen in the freezing medium with 30% FBS, FBS may also act as a protection against damage caused by freezing and thawing. These results suggest that freezing cells by the centrifugation

method inflicts damage on the fine structure of the cell surface necessary for cell attachment. As far as we examined, the addition of FBS gave definite protective effects on damage by freezing and thawing, although the exact mechanism was unclear.

In contrast to the conventional method, in the recommended method the cell attachment increased remarkably, and the effect of the serum addition on the cell attachment was not observed (Table 6). Thus, the viable, unattached cells (12%) frozen by the recommended method appear to suffer no damage to their attachment capability which can be reversed by serum. Often, part of cell damage ascribed to freezing and thawing is actually osmotic damage associated with the removal of an additive after thawing. It has been shown that slow dilution (Sloviter, 1951; Evans, Montes de Oca, Bryant, Schilling and Shannon, 1962) and step-wise dilution (Mazur, Farrant, Leibo and Chu, 1969) give higher survivals than the direct dilution. In this experiment, however, the recovery and the viability in these dilution methods were same as that in the recommended method (directly 5-fold dilution). We have not yet been able to raise further the attachment of viable cells.

Vol. 16 1981

The procedures for the preservation usually involve the addition of a cryoprotective agent, cooling at a constant rate to about −60°C to lose all freezable water, and storage at −196°C. However, McGann (1979) has recently reported that with penetrating agents (5% glycerol for 30 min at 37°C and 5% DMSO for 30 min at 0°C) recovery was maximal in samples cooled at 1°C/min to −30°C or lower followed by rapid cooling to −196°C and that for nonpenetrating agents (5% glycerol within 5 min at 0°C and 5% hydroxyethyl starch for 30 min at 0°C) maximum recovery was obtained when the cooling rate was controlled between −5 and −20°C. Cooling at 1°C/min to −60°C has been lethal in the presence of non-penetrating agents. In our procedure, the recovery was not different between samples cooled at 1°C/min to −25°C after pretreatment for 3 h at 4°C and that to −35°C, and increased rather in cells pretreated with glycerol at 4°C for 3 h than for 0, and 20 h (unshown data). In the practical method, therefore, the cells were cooled at 1°C/min to −35°C not to −60°C followed by direct immersion in liquid nitrogen after pretreating with glycerol for 3 h at 4°C for intracellular permeation of the additive.

The effects of other tissue culture media, such as Eagle's minimum essential medium (MEM) and McCoy 5a medium, on cell freezing and thawing were compared with that of BME in the presence of 10% FBS and 10% glycerol by using the centrifugation method. The results showed that the cell attachment was better in McCoy 5a medium (40.2%) than in BME (32.9%) and MEM (35.7%). The viability was not different between these media. However, cells frozen in BME with 30% FBS showed higher recovery than in these culture media with 10% FBS, and so the growth medium for the cultures can be used in practice as a base of freezing medium in the recommended method.

Our purpose in this paper is to develop the long-term storage technique of human diploid fibroblasts with glycerol, for using the cells as a tool for the research on cellular aging. Accordingly, young cells of early phase II were used in the present experiment. The recovery of cells frozen by the recommended method was then examined in the cells of different PD. Changes in the recovery of frozen cells associated with aging have been summarized in a subsequent paper (Kondo and Yamamoto, 1981). From the practical viewpoint of cell freezing, it has been revealed that the preservation of TIG-1 cells by freezing in the recommended method could be carried out under favorable conditions up to PD 61 (91% of lifespan).

SUMMARY

A practical method for long-term storage of human diploid fibroblasts was investigated. The conditions of increased cell attachment (recovery) and/or viability are as follows; freezing 1–10 × 10^6 cells/ampoule at a rate of 1°C/min to −35°C with the culture medium containing 30% FBS and 15% glycerol, thawing the contents rapidly by agitation in a 40°C water bath, and diluting 5-fold by the addition of the growth medium at 15 min after thawing. Consequently, this recommended method shows 78.8% recovery and 90.8% viability, in contrast to 32.9% recovery (82.5% viability) obtained in the conventional method. On the other hand, cells frozen with DMSO as an additive showed 52.9% recovery and 85.9% viability. The preservation of human diploid fibroblasts with glycerol was accordingly preferred to a method with DMSO. This practical method is useful for long-term storage of not only young cells but aged cells for the research on cellular aging.

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Vol.
16
1981

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EFFECTS OF ETHIDIUM BROMIDE ON DEVELOPMENT AND AGING OF *DROSOPHILA*: IMPLICATIONS FOR THE FREE RADICAL THEORY OF AGING

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Vol.
16
1981

INTRODUCTION

IT HAS been well established that ethidium bromide (EB), an interchelating dye, induces respiratory-deficient mutants in yeast mitochondria, with no apparent effect on the nuclear DNA (Slonimski *et al.*, 1968; Lloyd, 1974). These mutants are characterized by the absence of cytochromes ($a + a_3$) and b, inability to grow on nonfermentable energy sources and absence of respiration (Slonimski *et al.*, 1968). EB is the most potent substance known to produce these mutations and, in contrast to the acridines, it does not require cell multiplication for the transformation (Slonimski *et al.*, 1968).

More specifically, treatment of cells with EB results in the cessation of mitochondrial DNA synthesis, inhibition of mitochondrial DNA transcription (Fukuhara and Kujawa, 1970) and breakdown of pre-existing mitochondrial DNA (Goldring *et al.*, 1970; Perlman and Mahler, 1971). Furthermore, incubation with high concentrations for extended periods results in the appearance of cytoplasmic "petite" mutants that contain no detectable mitochondrial DNA (Nagley and Linnane, 1970; Williamson *et al.*, 1971; Michaelis *et al.*, 1971).

As regards gerontological research, Tudzynski and Esser (1977) have shown that EB in the culture medium at concentrations of 10^{-4}–10^{-5} M delays the onset of senescence in the ascomycete *Podospora anserina.* This observation is very interesting in relation to Harman's (1972) hypothesis that the maximal life span of organisms is an expression of genetic control over the rate of oxygen utilization by their mitochondria. Previous research from our laboratory suggests that, at least for the insect *Drosophila melanogaster* the rate of respiration may act as a biological clock setting the pace of senescent disorganization. In effect, adult flies maintained at 25°C showed higher oxygen consumption and accelerated loss of vitality (expressed in negative geotaxis and mating) than flies aging at 21°C. Exposure to the higher temperature was also associated with life shortening and high levels of lipid peroxidation in the tissues of *Drosophila,* probably induced by an increased production of free radicals at 27°C (Miquel *et al.*, 1976; cf. Sheldahl and Tappel, 1974).

The present article deals with further investigation of the role of oxygen consumption in determining the life span of *Drosophila.* Thus EB has been used in an attempt to produce respiratory-deficient mutants, which, in agreement with both the "rate-of-living" (Pearl, 1928) and the free radical theories of aging (Harman, 1956), have lower respiration rates and live longer than normal flies.

METHODS AND PROCEDURE

The flies used in these experiments were *Drosophila melanogaster* of the Orgeon R strain which have been inbred in this laboratory for 16 years. They were maintained at 25°C on "formula 4-24" instant *Drosophila*

medium (Carolina Biological Supply Co.) under constant light conditions. Ethidium bromide (EB) was procured from Aldrich Chemical Co. and added to the dry medium in solution at a concentration of 2×10^{-4} (0.0079% w/v). Previous dose–response analysis revealed that EB at a concentration of 2×10^{-5} had no effect on development time and that flies failed to develop on medium containing 2×10^{-3} M.

The eggs required for development studies were provided by females 7–14 days old. Eggs were laid during a 24-h period beginning at 9 a.m. They were immediately collected and carefully distributed in half-pint bottles containing the nutritive medium. Lints (1971) has shown that larval population density can drastically affect the duration of development as well as larval size and lifespan of the imago. Hence, this effect was minimized by placing exactly 50 eggs in each bottle containing equal volumes of culture medium.

The emerging adult flies were counted three times daily at 9 a.m., 12.00 p.m. and 4.00 p.m. and placed in 35 ml vials in groups of 25 flies per vial. At 7 days following emergence, flies were weighed individually on a Cahn model 7500 electric microgram balance. Statistical analysis of body weight and development time was performed, using the Student's *t*-test.

Males and females were separated and males were replaced in 35 ml vials at 25 flies per vial. Females were not used for life span studies. Transfer of male adults to fresh medium was performed three times weekly and mortality data recorded at time of transfer. Two series of non-simultaneous life span experiments were carried out at 27°C in order to evaluate the effects of EB on longevity: (1) life span of adults treated with 2×10^{-4} M EB during development compared with adults developed on control food (with both groups fed control food as adults); (2) life span of adults fed on EB compared with adults fed control food. These last two groups were developed on control food.

Oxygen consumption was determined by standard manometric techniques using groups of approximately 50 flies in 15 ml Warburg flasks. Manometer readings were made every 10 min and oxygen consumption was calculated from the readings from 10 to 100 min.

Electron microscopic studies were performed on 7-day-old adult flies, using 10 controls and an equal number of flies which were treated with 2×10^{-4} M EB during development. The thoracic flight muscle was processed as previously described (Rockstein *et al.*, 1975) and epon-araldite sections were examined with a Philips 300 electron microscope.

RESULTS AND DISCUSSION

EB at a concentration of 2×10^{-4} M in the medium increased the development time of *D. melanogaster* by 32% (Table 1). Mean weights of EB-treated flies were 88% of the controls for males and 77% for females (Table 2).

TABLE 1. EFFECT OF ETHIDIUM BROMIDE ON THE DEVELOPMENT OF *Drosophila melanogaster*

Treatment	% flies eclosed	Mean development time (h)	S.D.	Range of development time (h)
Ethidium bromide	40	469.7	± 31.3	432.5–538.5
Control	56	355.7	± 30.7	334.5–443.5

Student's *t*-test: $t = 12.58$; $p < 0.01$.
% increase in mean development time: 32.

TABLE 2. EFFECT OF ETHIDIUM BROMIDE ON THE BODY WEIGHT OF *Drosophila melanogaster*

Treatment	Mean male weight (mg)	S.D.	Mean female weight (mg)	S.D.
Ethidium bromide	0.67	± 0.03	0.82	± 0.06
Control	0.76	± 0.05	1.07	± 0.08

Student's *t*-test: males $t = 4.03$, $p < 0.01$; females $t = 7.50$, $p < 0.01$.

Both the mean and the maximum life span of flies which developed on medium containing 2×10^{-4} EB were increased by 21.5 and 17.9% respectively when fed control food as adults (Fig. 1). Furthermore, flies allowed to develop on control food had both mean

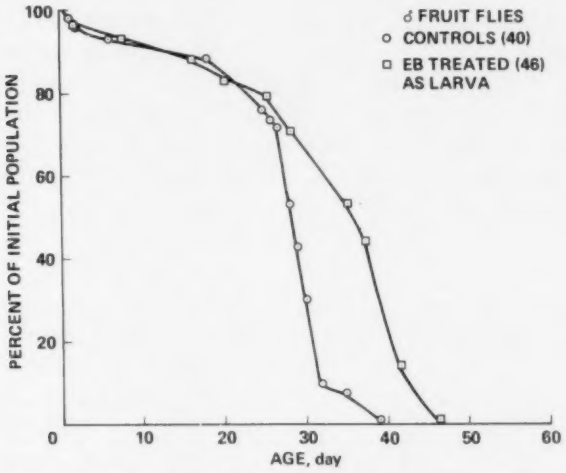

FIG. 1. Survivorship curves of control *Drosophila* and of flies which were treated with ethidium bromide (EB) during development and were maintained on normal nutritive medium during their adult life.

Vol. 16 1981

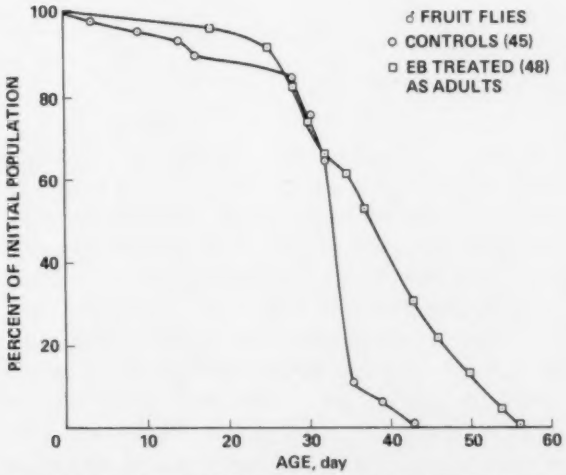

FIG. 2. Survivorship curves of control *Drosophila* and of flies which developed on normal medium and were maintained on EB-supplemented medium during their adult life.

and maximum life span increased by 22.6 and 30.2% respectively when fed EB as adults (Fig. 2).

The oxygen consumption of the flies treated with EB during their adult life was considerably lower than that of control flies of the same age (Table 3). Administration of EB during development also resulted in lower respiration values. However, in this last instance the decrease in oxygen consumption was not statistically significant (Table 3).

By electron microscopy, the wing muscle of the control flies showed a normal appearance (Fig. 3), except for an occasional swollen mitochondrion. These abnormal mitochondria were much more abundant in the flies which developed on the EB-medium (Fig. 4).

In our opinion, the above results are in agreement with the view that aging may be induced, at least in part, by free radicals produced in mitochondria during the course of normal oxidative phosphorylation (Harman, 1956, 1972). That free radicals and their resulting products (lipid peroxides in particular) are indeed formed in mitochondria has been recently shown by Nohl and Hegner (1978). According to these authors, a constant fraction of 20% of the radicals produced during respiration escape quenching by intramitochondrial superoxide dismutase and can therefore induce peroxidative degradation of the lipids of the inner membrane.

TABLE 3. EFFECTS OF ETHIDIUM BROMIDE ON THE OXYGEN CONSUMPTION OF MALE ADULT *Drosophila melanogaster*, OREGON R (μl/mg BODY WEIGHT/24 h)

Experiment No.	Control (*N*)	Developed on ethidium bromide (*N*)	Treated with EB as adults (*n*)
1	78.7 (35)	77.4 (37)	65.6 (33)
2	72.8 (38)	58.9 (48)	61.4 (49)
3	75.8 (32)	68.8 (44)	68.8 (42)
4	68.4 (32)	66.8 (36)	65.8 (37)
Means	73.9	67.9	65.4
S.D.	± 4.4	± 7.5	± 3.0
		$t = 1.36$	$t = 3.18$
		N.S.	$p < 0.05$

In view of the above, it is not surprising that treatment with EB results in an increase in the life span of *Drosophila*. The lower oxygen consumption of the treated flies (Table 3) suggests that, as is the case for yeast (Slonimski *et al.*, 1968; Lloyd, 1974), this interchelating dye disorganizes the respiratory apparatus of *Drosophila* cells. Most likely, the EB-treated flies sustain damage to their mitochondrial genome, with the result that respiration is depressed because of decreased ability to synthesize respiratory enzymes (cf. Slonimski *et al.*, 1968). Since less energy can be obtained to support growth and cell differentiation, the development time is lengthened and stunted flies are produced (Tables 1 and 2). On the other hand, in agreement with Harman's concept that senescence may result from the side effects of respiration-linked free radicals, the EB-induced depression of aerobic metabolism is apparently accompanied by a decreased rate of ageing. Thus, our results are in agreement with the "rate-of-living" theory, which was first proposed by Pearl (1928) and by Alpatov and Pearl (1929). According to these authors, *Drosophila* has a longer duration of life at lower temperature because it has a lower "rate-of-living": in other words, it is less active. Previous work from our laboratory has confirmed that the decreased rate of aging of flies maintained at lower temperature is associated with a decreased rate of oxygen utilization. Therefore, it is reasonable to assume that flies living at lower temperature have lower respiration rates and this, in turn, results in decreased formation of senescence-inducing free radicals.

In summary, our work suggests that the life span of fruit flies can be influenced both by environmental and by genetic interference with the rate of oxygen consumption. Since, apparently, the injury induced by free radicals cannot be fully repaired, the mitochondrion may very well be the site of intrinsic senescent change.

Vol.
16
1981

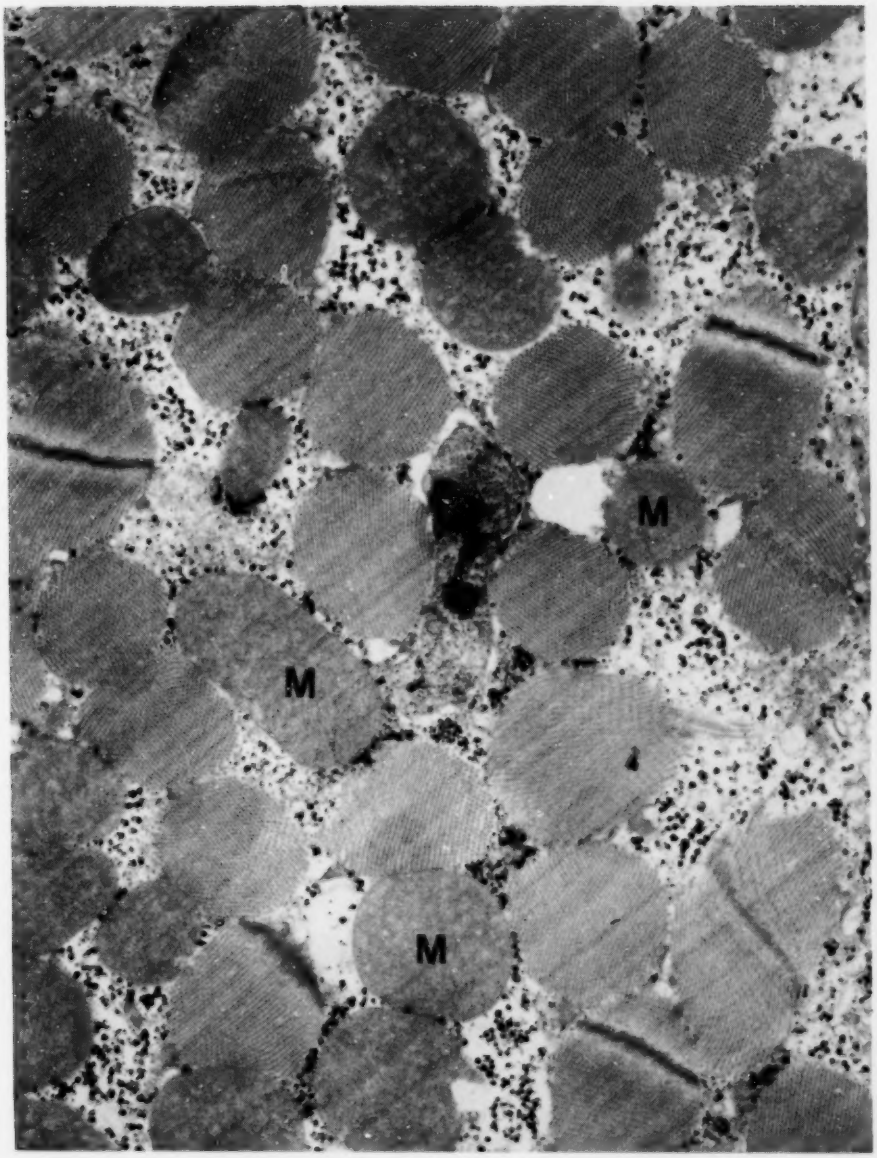

FIG. 3. Normal flight muscle from the thorax of a 7-day-old adult fly which developed on normal (non EB-supplemented) medium. The mitochondria (M) show a perfectly normal fine structure. × 13,650.

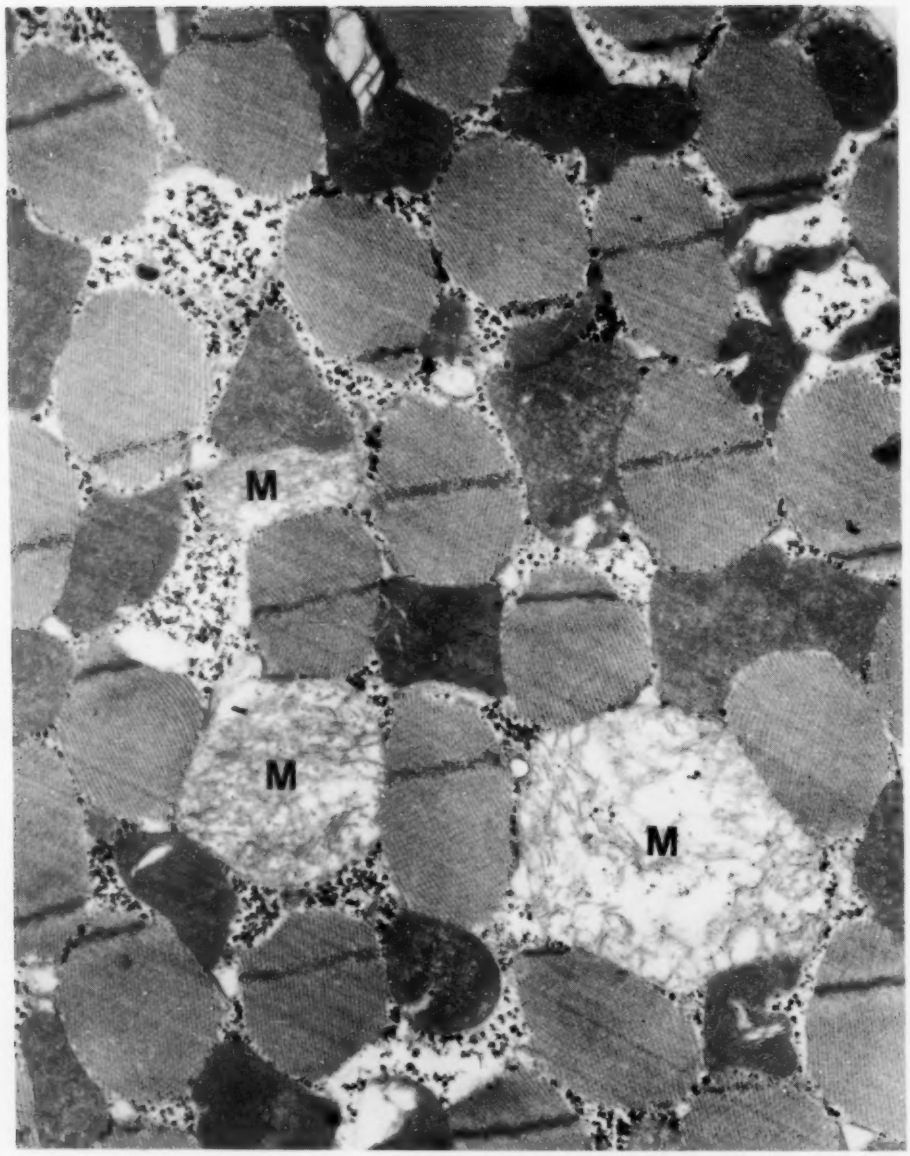

FIG. 4. Flight muscle from the thorax of a 7-day-old adult fly which developed on medium containing EB. Notice the abundant swollen mitochondria (M) with disorganized cristae. × 31,650.

Vol
16
198

SUMMARY

Ethidium bromide (EB) at a concentration of 2×10^{-4} M in the medium increased the development time of *Drosophila melanogaster* by 32%. Concentrations of 2×10^{-5} had no effect on development and flies failed to develop in media containing 2×10^{-3}. The adult body weights of the flies which developed on the EB-medium were 80% of the controls for the males and 77% of the controls for the females. Both the mean and the maximum life span of the flies which developed in medium containing EB were increased by 21.5 and 17.9% respectively when fed control food as adults. Furthermore, flies allowed to develop in control food had both mean and maximum life span increased by 22.6 and 30.2% respectively when fed 2×10^{-4} EB as adults. The oxygen consumption was lower for both flies treated with EB during their development or during their adult life.

The results suggest that EB, which has been shown to produce respiratory mutants in yeast, has a similar disorganizing effect on the respiratory apparatus of *Drosophila*. The fact that this mitochondria-induced lowering of respiration results in increased life span supports the "rate-of-living" theory of aging and Harman's concept that senescent disorganization is linked with the release of free radicals in the mitochondria during the course of normal aerobic metabolism.

Vol. 16 1981

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AGE-DETERMINATION IN THE TROPICAL AGAMID GARDEN LIZARD, *CALOTES VERSICOLOR* (DAUDIN), BASED ON BONE HISTOLOGY

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Vol.
16
1981

INTRODUCTION

THE IMPORTANCE of age determination in ecological, demographical and gerontological studies and in research concerning prehistoric material has been realized as a basic necessity during recent years.

In species capable of breeding in captivity, the young can be reared under standard laboratory conditions and their ages determined by keeping the birth record. But for others which breed favourably in nature, suitable alternative methods to evaluate the age of individuals are required. Morphometric data such as body weight, snout-to-vent length, dental criteria (eruption or wearing of teeth) and gonad weight, though they have been successfully employed for indirect measures of chronological age, are often subject to a criticism of doubtful reliability. But the use of bone histology, based on 'skeleto-chronological' techniques dealing with hard tissues of vertebrates in general (Castanet, 1974, 1975, 1978), has been recognized as the most meaningful and practicable method not only to assess individual age, but also the speed of growth, the age of sexual maturity and the longevity of various species. In most ectotherms, apart from remodelling changes causing the occurrence of bands in cortical bone, a second pattern of bone deposition occurs in successive layers: thin and thick, corresponding with 'slow' and 'active' growth of individuals during colder and warmer seasons respectively. Such cyclical growth marks are considered to be the expression of annual growth periodicity. The actual age can be determined by counting the annual rings in the compact bone of the cortex (Saint Girons, 1965). 'Growth lines' are also found in scales and the skeleton (cross section of spines of fins) of fish (Qasim and Bhatt, 1966; Bhatt, 1969; Semakula and Larkin, 1968) and in vertebral and pleural shields of turtles (Cagle, 1946; Sexton, 1959). Cross-sections of diaphysis of long bones (femur and humerus) of amphibians (Griffiths, 1962; Smirina, 1972; Castanet, 1975; Smirina and Rocek, 1976), lizards (Peabody, 1961; Warren, 1963; Smirina, 1974; Castanet, 1978) and turtles (Castanet and Cheylan, 1979), and flattened areas of different bones (mandible, ectopterygoid and neural spine) of snakes (Bryuzgin, 1939; Petter-Rousseaux, 1953; Peabody, 1961; Castanet, 1974; Castanet and Naulleau, 1974; Saint Girons, 1975) have been shown to exhibit conspicuous growth marks which could be successfully employed for age-determination in respective species. These marks are variously termed as 'cortical banding' or 'cortical lamination' (Enlow, 1969); 'resting lines' or 'adhesion lines' and as 'lignes d'arrêt de croissance' (LAC) (Castanet, 1978).

Even though extensive studies have been done on bone histology of a variety of temperate species of reptiles, the two main aspects that have been envisaged in the present study might be of considerable importance. One of these aspects is the problem of whether a tropical species would show a similar pattern of zonation of osseous tissue as in temperate

species, in view of the point raised by Enlow (1969) that climatic and geographical differences might influence the nature of cortical lamination. The other aspect concerns a correlation between size (age) and cortical lamination using a complete range of individuals from juvenile to old, as due consideration has not so far been given for such a detailed study in reptilian species (Enlow, 1969).

With these premises in mind, the present study concerns the histology of long bones (humerus, femur), phalanges and epiphyses of the most commonly available tropical agamid garden lizard, *Calotes versicolor*.

MATERIALS AND METHODS

Male garden lizards, *Calotes versicolor* Daudin (Agamidae), of various sizes used in the present study were collected from Berhampur localities between January and April. These lizards breed during the summer and the eggs laid by gravid females have an incubation period of about 45 days. Hatchlings mostly appear in late September. However, lizards of various sizes are available in the wild from late September to late April of the following year.

Due to non-availability of practical facilities no attempt has been made in the present study to use the microradiography or fluorochrom techniques of Castanet (1978). However, the methods followed for bone histology were essentially the same as those used by Castanet (1978), but with minor modifications.

Following an acclimation period of about 7 days in the laboratory the lizards were killed by a blow on the head. The long bones (humerus and femur) and the phalanges of the hind limbs were fixed and preserved in 70% alcohol. Decalcification was carried out at room temperature (28 ± 2°C) in 10% versenate (EDTA, disodium salt, BDH) solution as described by Birge and Imhoff (1952). The period of decalcification lasted for 20–40 h, depending on the size of the lizard from which the sample was taken. After decalcification the materials were washed thoroughly with distilled water, dehydrated, cleared and processed for paraffin (Merck, 56–58°C) block preparation using the standard procedure. Serial sections (25–30 μ) were cut using a rotary microtome. Delafield haematoxylin was used to stain the sections.

Even though descriptions of only six lizards have been presented in this communication (Table 1), the bones of a number of lizards belonging to three different age-groups have been processed. Lizards of the same morphometric parameters exhibited an almost coherent picture.

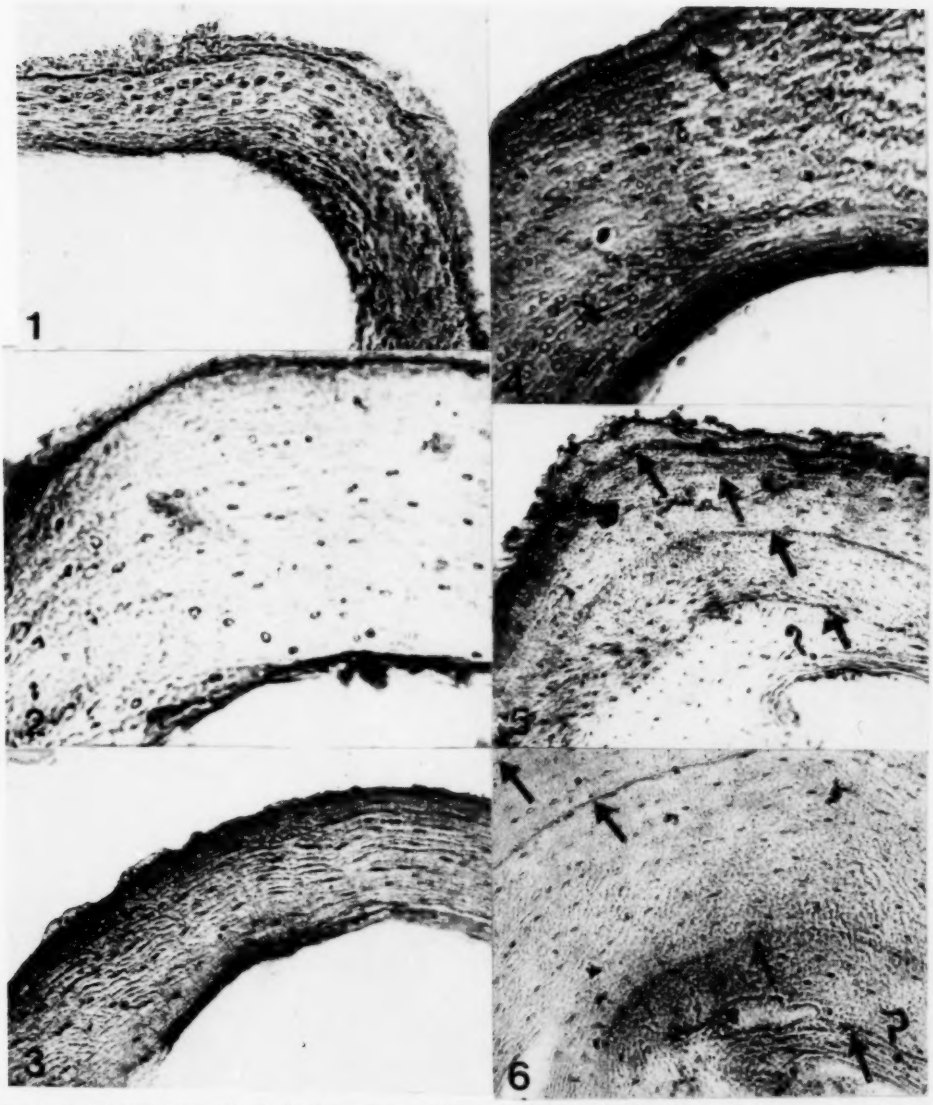
RESULTS

Histological structure

As in many other species, the histological structure of long bones of the garden lizard present a typical lacertian 'lamellar-zonal pattern' (de Riqulés, 1976). Cross-sections of shaft (Figs. 1–12) reveal that it is composed of dense lamellar and avascular bone of periosteal origin. In some sections one may find deposition of endosteal bone in the inner border of the periosteal cortex. The very simple nature of the bone structure presents a favourable condition for the observation of growth lines (LAC) in the cortical layer.

The osteocytes are confined to the thicker zone of the cortex, whereas the thinner annulation (LAC) is devoid of cellular elements. The nuclei of osteocytes and the thinner annulations are haemotoxylinophilic. In young specimens the osteocyte size is larger than in old specimens (Figs. 13 and 14). The nuclei are almost centrally placed in the young but they occupy an eccentric position in old lizards possibly due to reduction in the volume of cytoplasm. The relative density of osteocytes in cortical bone seems to be greater in the young than in the old.

Histological sections of humeral epiphyses revealed a spongy cartilaginous structure with hyaline cells in the young (Fig. 16), but in the old the epiphyses get calcified, the bony epiphyses overhang the shaft by lappets and ultimately unite with it (Fig. 17).



FIGS. 1–6. Photomicrograph of the transverse sections (×165) of the diaphysis of the humerus of garden lizards of various age-classes.

FIG. 1. Young immature male (body wt 7 g; S–V length 70 mm). There is no indication of a complete LAC.

FIG. 2. Sexually mature male (body wt 24 g; S–V length 96 mm). There is no development of LAC.

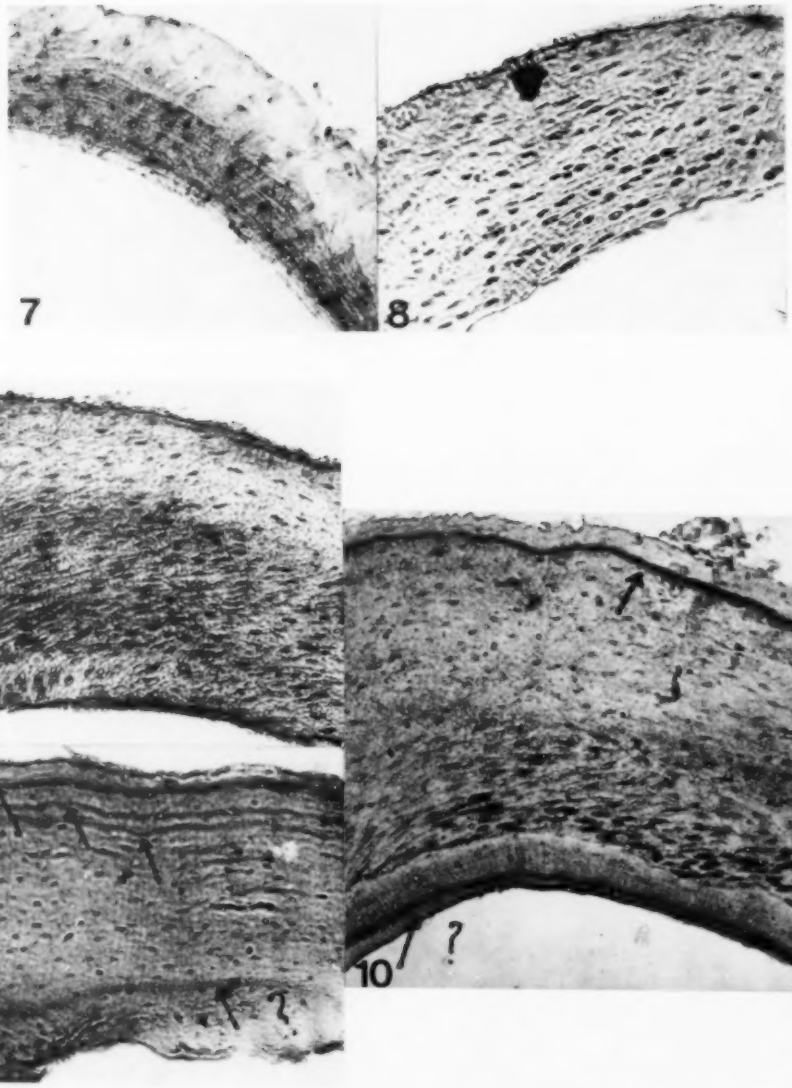
FIG. 3. Sexually mature male (body wt 34 g; S–V length 105 mm). There is still no development of LAC.

FIG. 4. Fully mature male (body wt 50 g; S–V length 118 mm). There is one complete LAC (indicated by arrow) near the periphery of the cortex.

FIG. 5. Old male (body wt 70 g; S–V length 125 mm) showing 4 (3?) LACs. Since the innermost LAC is not very clear a question mark has been put.

FIG. 6. Another example of an old male (body wt 76 g; S–V length 128 mm) showing 4 (3?) distinct LACs.

Vol.
16
1981



FIGS. 7–12. Photomicrographs of transverse sections (× 165) of diaphysis of the femur of garden lizards of various age-classes.

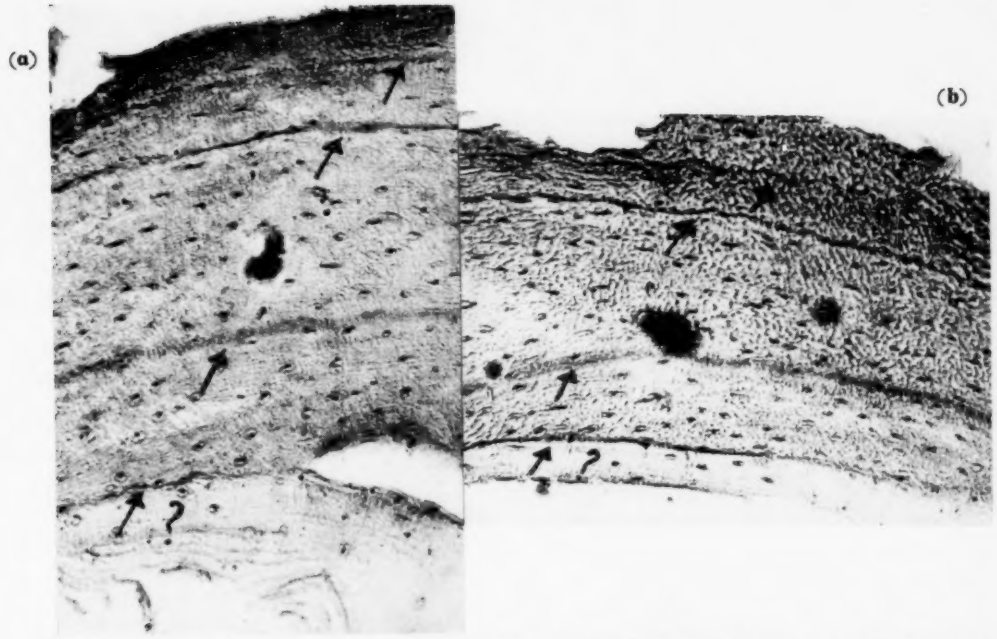
FIG. 7. Young immature male, as described in Fig. 1. As in the humerus the development of LAC is lacking.

FIG. 8. Sexually mature male, as described in Fig. 2. As in the humerus there is no development of LAC.

FIG. 9. Sexually mature male, as described in Fig. 3. There is still no development of LAC.

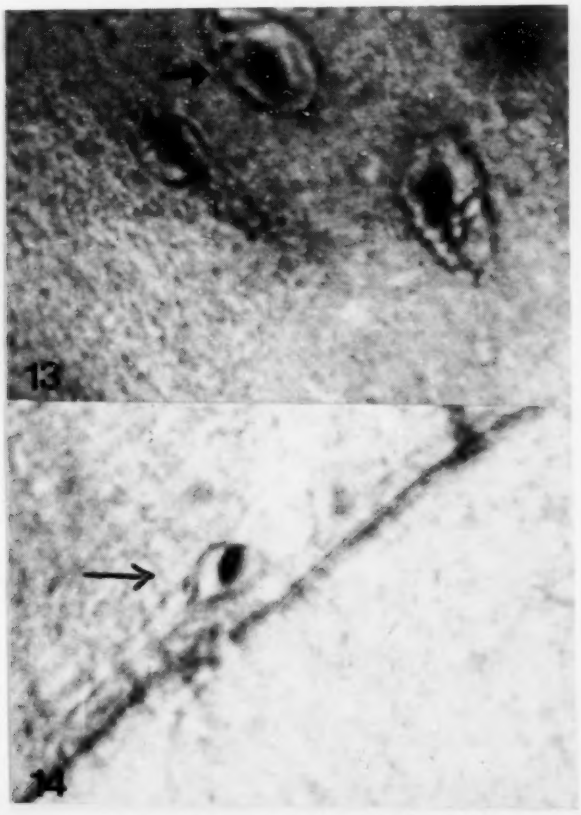
FIG. 10. Fully mature male, as described in Fig. 4. There is one complete LAC near the surface of the cortex. An indication of developing LAC in endosteal bone has been question-marked.

FIG. 11. Section of an old male, as described in Fig. 5, showing 4 (3?) LACs.

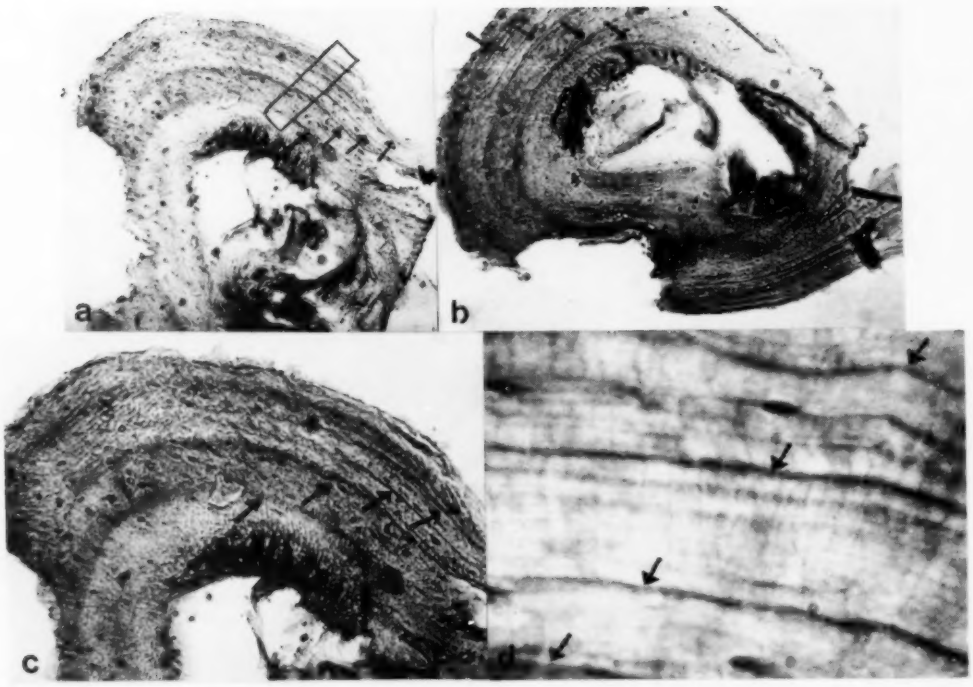


FIGS. 12a, b. Two separate sections of another old male, as described in Fig. 6, showing 4 (3?) distinct LACs. In Fig. 12a the white area in right hand margin indicating a furrow is an artifact caused by tearing of the section during preparation.

Vol.
16
1981



FIGS. 13, 14. Photomicrographs of enlarged transverse sections (× 1650) of humerus of a young lizard (body wt 12 g; S–V length 84 mm) and an old lizard (body wt 70 g; S–V length 125 mm) respectively, showing osteocytes in the cortex.



FIGS. 15a–c. Photomicrograph of two separate transverse sections (×165) through a phalange of a hind limb of an old male, showing 4 LACs in each. Fig. 15c is an enlarged portion of Fig. 15a. Some LACs show double lining.

FIG. 15d. Photomicrograph of a magnified view (×1650) of Fig. 15a, showing 4 distinct LACs. The exact level of magnification in 15a has been indicated by a rectangular mark.

Vol.
16
1981

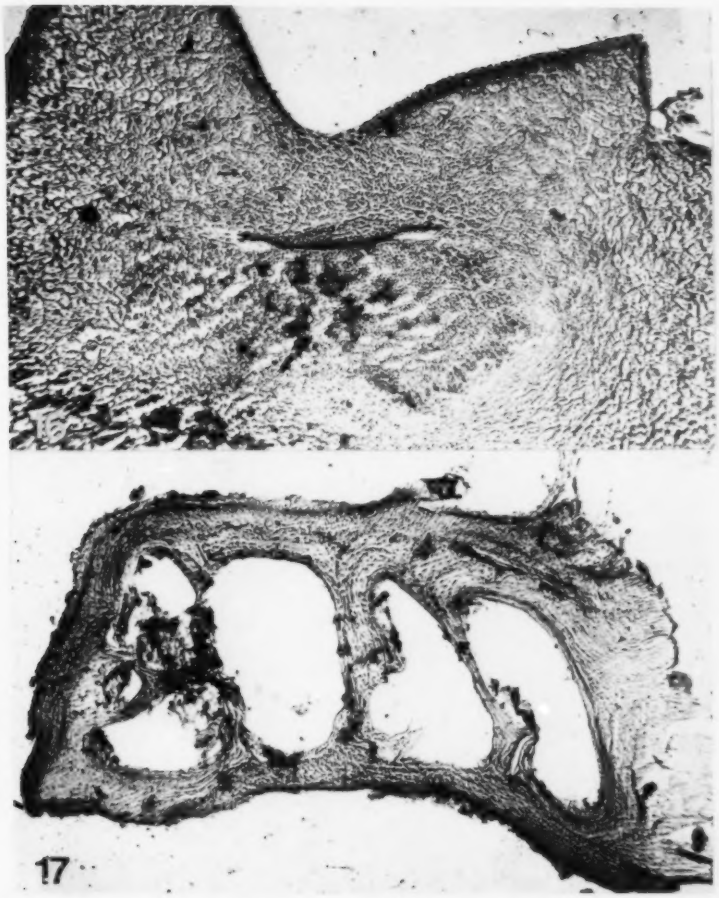


FIG. 16. Photomicrograph of a transverse section through a humeral epiphysis of a young lizard, as described in Fig. 1. (Entire section × 66.)

FIG. 17. Photomicrograph of a transverse section through a humeral epiphysis of an old lizard, as described in Fig. 5. (Entire section × 66.)

Growth marks in long bones and phalanges

Identification of LAC using 'skeletochronology' is likely to be confused due to the presence of double or triple lines, endosteal bone or resorption and removal of some previously deposited ones (Enlow, 1969; Castanet, 1978).

In cross-sections of long bones (humerus and femur) of the garden lizard the LACs are chromophilic (haemotoxylinophilic) and appear regularly as concentric rings (Figs. 1–12b), similar to the types observed earlier in temperate species of amphibians, lizards and turtles.

Cross-sections of phalange also present a similar picture, but some of the LACs appear to be double lined (Figs. 15a–d).

Age-determination on the basis of LAC

Cross-sections of humerus and femur of a complete age series reveal that in young, immature and sexually mature individuals (the offspring of the same breeding season who have not yet completed a year in the wild) there is no development of a distinct and complete LAC. But in fully mature lizards there is one LAC and in old, three or four. The number of LAC in the humerus and femur of the same individual was fairly consistent (Figs. 1–12b, Table 1). Similarly, the number of LAC in cross-section of phalange (Figs. 15a–d) correlated well with the number in cross-section of diaphysis of long bones.

Based on the number of LAC, the data presented in Table 1 indicate the age of individual lizards, the representatives of three different age-groups based on the criteria of morphometric observations. Since the old specimen used in the present study (body wt 76 g) was the largest male garden lizard available in Berhampur localities for the last 11 years, it was also considered to be the oldest. Since this lizard exhibited 4 LACs in its skeleton, it seems that the longevity of male members of *Calotes versicolor* in the wild is 4 years.

Vol. 16 1981

DISCUSSION

'Skeletochronological' techniques, including bone histology, microradiography and fluorochrom labellings have been extensively used not only for ascertaining the histological pattern of bone, but also to determine the age of individuals by counts of 'growth lines' (LAC) mostly in temperate species of lizards, snakes and turtles (Castanet, 1974, 1978; Castanet and Cheylan, 1979). Some authors (Cagle, 1948; Enlow, 1969; Zangerl, 1976) have raised the point that since growth rings are related to differences in the temperature-dependent seasonal metabolic activity, they are more pronounced in temperate climates than in the tropics. This led us to undertake the present study for a comparison between the temperate and tropical species. As far as we are aware, the present observation of garden lizards is the first detailed report on a tropical species, even though Griffiths (1962) and Warren (1963) have used some reptiles of equatorial and tropical regions.

The bone histology of the garden lizard confirms that it has a 'lamellar-zonal pattern' and is avascular (de Ricqlés, 1976; Castanet, 1978), suggesting that the basic histological pattern is similar in species belonging to the subclass, Lepidosauria, whether temperate or tropical.

The relative density of osteocytes in cortical bone of old lizards is found to be sparse compared to that of young, immature lizards. That the distribution of osteocytes may be age-dependent in turtles has been previously reported (Suzuki, 1963). Alteration in shape

TABLE 1. NUMBER OF GROWTH LINES (LAC) IN THE CROSS-SECTIONS OF BONE OF MALE GARDEN LIZARDS OF VARIOUS AGE-GROUPS

Age group	Range of body wt (g)	Range of S–V length (mm)	Range of tail girth (mm)	Range of gonad wt during non-breeding season (mg)	Present representatives							
					Wt (g)	Length (mm)	Tail girth (mm)	Gonad wt (mg)	LAC Humerus	LAC Femur	Phalange	Age (years)
Young	5–15	64–85	15–29	1–5	7	70	21	1	0	0	Not carried out	Under one year
Middle-aged	20–40	93–114	32–41	6–35	24	96	35	6	0	0	Not carried out	Under one year
					34	105	39	35	0	0	Not carried out	Under one year
Old	45–76	115–127	45–60	43–60	50	118	45	43	1	1	Not carried out	1–1.5
					70	125	56	78	4 (3?)	4 (3?)	Not carried out	3–4
					76	127	60	—	4 (3?)	4 (3?)	4	3–4

and size of the osteocytes and the appearance of the eccentric positions of nuclei within the lacuna, possibly due to reduction in the volume of cytoplasm, may indicate a stage of cell degeneration in old age, as has been reported for *Iguana* (Enlow, 1969). However, this aspect of the present study needs further elaboration.

On the basis of bone histology, it seems that in cortical bone the development of thicker and thinner annulations, casued by 'active' and 'slow' phases of temperature-dependent growth, is a characteristic of both temperate and tropical species, the climatic and geographical differences between them notwithstanding. The demarcation between winter and summer in Berhampur localities, which are about 10 km away from the sea-shore (Gopalpur-on-Sea), is not as well defined as in a temperate climate, but this does not preclude the value of LAC as an indicator of individual age in garden lizards. This confirms the findings of Griffiths (1962) that similar patterns of growth rings appear in the skeleton of both temperate and tropical species of snakes.

Castanet (1978), using a variety of temperate species of lizards, has confirmed that the recurrence of the LAC in lacertians shows an annual regularity and thus the number of LACs can be used as a suitable indicator of age in lizards. This seems to be true for the garden lizards used in the present study and is anticipated to be the case of other species of tropical reptiles.

Vol. 16 1981

On the basis of histological data we confirm that in young, immature and sexually mature lizards, which are offspring of the same breeding season and have not yet completed a year in the wild, there is no development of skeletal growth marks that have annual growth periodicity. Since the period of hatching of these lizards in the wild extends from September to February it is possible to predict the age of the young, immature lizards of the present study as 2–3 months and that of sexually mature lizards as 5–9 months. The 10:6.6 Ca/P molar ratio of bone, a suitable parameter to assess the extent of calcification, was shown to be reached in sexually mature (25 g body wt) lizards (Sahu, Mishra and Patnaik, 1973) and maintained thereafter. It is still not clear if the phenomenon of calcification has any correlation with the development of growth marks in the skeleton of garden lizards.

Counts of LAC provide evidence for the fact that the longevity of male garden lizards in the wild is about 4 years. The calcification and the bony union of humeral epiphyses with the shaft in old lizards suggests no further growth as fusion of epiphyseal centres has been considered a mechanism for limiting growth (Haines, 1969).

The present observations on age-determination based on 'skeletochronology' correlate extremely well with our previous method of age-grouping of lizards using the criteria of morphometric data (Rao and Patnaik, 1973; Mishra and Patnaik, 1974; Panigrahy and Patnaik, 1974; Padhi and Patnaik, 1976, 1978; Kara and Patnaik, 1980; Patnaik, 1981). But while the morphometric data presented an indirect measurement of chronological age, the histology of osseous tissue accurately measured the ages of the lizards, thus highlighting the value and wide applicability of the technique of 'skeletochronology' in comparative histological studies. It would be worth extending such studies to a variety of tropical reptiles, which we intend to pursue in the future.

SUMMARY

Male garden lizards showed excellent positive correlations between body weight and S–V length; body weight and tail girth; and body weight and gonad weight, suggesting the utility of these parameters as indirect markers of chronological age. However, the

reliability of such morphometric data needed rapport from a more accurate method of bone histology based on the techniques of 'skeletochronology'. Cross-sections of diaphyses of the humerus and femur bones of lizards of a complete age series revealed that while in young, immature and sexually mature lizards who have not yet completed a year in the wild there are no distinct annulations, the representative of fully mature and old lizards exhibited an alternation of thinner and thicker annulations arranged concentrically. Cross-sections of phalanges of old lizards also showed agreement with the annulations in diaphysis. The thinner annulations (LAC), corresponding with the period of slow growth, as in temperate species of lizards, were haemotoxylinophilic (chromophilic) and showed yearly growth patterns, their number representing the age of the lizards in years.

Transverse sections of humeral epiphysis of old lizards exhibited, in contrast to the cartilaginous nature of young immature lizards, an almost complete calcification and bony union, suggesting a limitation of further growth. Thus, based on the recurrence of growth lines (LAC) in the diaphysis of long bones and in the phalanges, it is possible as in temperate species of lizards and turtles, to determine accurately the age of individuals belonging to a wild population of tropical lizards.

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Vol.
16
1981

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PLASMA TRIIODOTHYRONINE RESPONSE TO THYROTROPIN RELEASING HORMONE, THYROTROPIN AND PROPRANOLOL IN OLD AGE

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Vol.
16
1981

INTRODUCTION

INVESTIGATIONS of the endocrine system with a special regard to the thyroid gland in old subjects have had an outstanding place in gerontological examinations, probably because of the previously hypothesized similarity of hypothyroidism to ageing.

Since a relativeiy reliable determination of the hormones has become possible by the radioimmunological methods, many authors have investigated the thyroid hormone levels and hormone metabolism in old people.

Most, but not all, authors report that thyroxine (T_4) levels do not differ in old age (Kind and Gosh, 1976; Jeske and Thorner, 1977; Olsen *et al.*, 1978; Wagner *et al.*, 1979). Also, the average serum triiodothyronine (T_3) levels were lower and the reverse triiodothyronine (rT_3) levels higher in old age, as compared to the values obtained in the case of younger subjects) Nicod *et al.*, 1976; Rudorff *et al.*, 1976; Burrows *et al.*, 1977). Most authors explain this by a shift of peripheral T_4 monodeiodination from T_3 to rT_3 (Faber *et al.*, 1976; Rudorff *et al.*, 1976; Vosberg *et al.*, 1976; Wenzel and Horn, 1976; Burrows *et al.*, 1977), but Frolkis and Valueva (1978) came to another conclusion, based on experiments with rats of different ages. They found an enhanced T_4 deiodination in the tissues, resulting in a nonvariable tissue content of T_3, alongside with a decrease of the functional activity of the thyroid gland with ageing.

To support the previous findings, the T_3 response to thryotropin-releasing hormone (TRH), thyrotropin (TSH) and propranolol and the suppressibility of TSH response to TRH by T_3 were investigated in old, healthy subjects.

MATERIALS AND METHODS

Ninety-six people aged 70–90 yr (41 males, 55 females) and 80 people aged 20–40 yr (36 males, 44 females) were examined. The gerontological group consisted of patients living in rehabilitation units either for social reasons or because they were suffering from chronic locomotory diseases. In the juvenile group were people who were examined for their vegetative complaints, but no actual disease had been found. Consent was obtained from each patient after a full explanation of the purpose and nature of the procedures to be used.

The following blood hormone levels (fasting values) were determined (Table 1) by commercially available KITs: T_4 by the competitive protein binding Thyopac-4 method; T_3 by the Amersham radioimmunoassay; rT_3 by the Biodata rT_3-KIT; TSH by the Byk-Mallincrodt RIA.

In addition to the fasting values in 19 old and 9 young subjects (Table 2) the responses of thyroid hormones to TSH were investigated: 10 U TSH (Ambinon) were administered intramuscularly and blood samples for T_4 and T_3 were taken before, and 4 h, 24 h and 48 h following the injections.

In 19 old and 14 young people, TRH was repeatedly given orally for four consecutive days at a dose of 20 mg (Richter Gedeon) daily at 8.00 a.m. Before administering the first dose, and also at 12.00 p.m., blood samples were examined for TSH, T_3 and, occasionally, T_4 each day (Table 3).

TABLE 1. PLASMA T_4, T_3, rT_3 AND TSH LEVELS IN DIFFERENT AGES

	T_4 Thyopac-4 (normal range = 51–160 nmole/l)	T_3 Amersham (normal range = 1.23–3.85 nmole/l)	rT_3 Biodata (normal range = 0.13–0.54 nmole/l)	TSH-RIA Byk–Mallincrodt (normal range = 0.0–3.8 μIU/ml)	Occurrence of $T_3 < 1.23$ nmole/l	Average TSH in cases of $T_3 < 1.23$ nmole/l
Aged 70–90 yr						
S.E.M.	121.1 ± 2.52	1.80 ± 0.07	0.44 ± 0.02	1.89 ± 0.23	32.3%	1.95± 0.21
n	68	96	57	70	31	23
Aged 20–40 yr						
S.E.M.	118.6 ± 3.6	2.45 ± 3.6	0.35 ± 0.05	2.28 ± 0.19	6.3%	—
n	33	80	30	64	5	
Significance	n.s.*	$p < 0.001$	$p < 0.02$	n.s.		

*No significance.

TABLE 2. PLASMA T_4 AND T_3 RESPONSE TO TSH IN DIFFERENT AGES

	T_4 (nmole/l)				T_3 (nmole/l)			
	Before TSH	After TSH (Ambinon 10 U i.m.†) 4 h	24 h	48 h	Before TSH	After TSH (Ambinon 10 U i.m.) 4 h	24 h	48 h
Aged 70–90 yr								
S.E.M.	117.58 ± 4.25	134.65 ± 8.95***	182.67 ± 8.95***	169.47 ± 9.11***	1.81 ± 0.18	2.49 ± 0.25**	2.40 ± 0.25*	2.41 ± 0.26*
n = 19								
Aged 20–40 yr								
S.E.M.	110.38 ± 5.17	137.70 ± 10.4**	178.7 ± 13.73***	157.01 ± 0.17***	2.19 ± 0.26	2.66 ± 0.27**	2.87 ± 0.35	2.79 ± 0.23*
n = 9								
Significance	n.s.‡	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Compared to the initial value:
*$p < 0.05$;
**$p < 0.01$;
***$p < 0.001$.
†Intramuscularly.
‡No significance.

Vol. 16 1981

TABLE 3. TSH AND T_3 RESPONSE TO REPEATED ORAL TRH IN DIFFERENT AGES

		4 × 20 mg oral TRH administration							
	T_3 before TRH administration (nmole/l)	4 h after the 1st bolus Δ TSH (μIU/ml)	4 h after the 1st bolus T_3 (nmole/l)	4 h after the 2nd bolus Δ TSH (μIU/ml)	4 h after the 2nd bolus T_3 (nmole/l)	4 h after the 3rd bolus Δ TSH (μIU—mi)	4 h after the 3rd bolus T_3 (nmole/l)	4 h after the 4th bolus Δ TSH μIU/ml)	4 h after the 4th bolus T_3 (nmole/l)
Aged 70–90 yr S.E.M. $n = 19$	1.52±0.23	8.81±1.25***	2.57±0.38*	2.72±0.42***	2.83±0.45	0.57± 0.32	1.79±0.29	1.19±0.62	1.97±0.26
Aged 20–40 yr S.E.M. $n = 12$	2.20±0.18	16.5±6.51*	3.32±0.35**	2.69±0.40***	3.63±0.44**	2.14±0.80*	3.43±0.37*	−0.1±0.35	3.37±0.37*
Significance	$p < 0.05$	n.s.	n.s.	n.s.	n.s.	< 0.05	$p < 0.01$	n.s.	$p < 0.02$

Compared to the initial value:
*$p < 0.05$;
**$p < 0.02$;
***$p < 0.001$.
†n.s.: no significance.

In 14 old subjects the blood T_3 and the TSH response were measured 4 h after oral administration of 20 mg TRH. After a month the blood T_3 was measured again and 2 × 5 μg T_3/day (Liothyronin) was administered for another 2 weeks, followed by a second TRH test and T_3 determination (Table 4).

TABLE 4. T_3 LEVELS AND TSH RESPONSE TO TRH BEFORE AND AFTER T_3 ADMINISTRATION IN 70–90-YEAR-OLD SUBJECTS

	T_3 (nmole/l)		TSH response to TRH (ΔTSH μIU/ml)	
	Before T_3 administration	After T_3 administration	Before T_3 administration	After T_3 administration
S.E.M. $n = 14$	1.50 ± 0.21*	1.40 ± 0.13*	8.81 ± 1.52	4.79 ± 2.06†

*Compared to the control 20–40-year-old subjects $p < 0.001$.
†Compared to the initial value $p < 0.05$.

Propranolol was given daily (3 × 40 mg) to 18 old and 12 young subjects for a week and the blood T_4, T_3 and rT_3 was measured before the administration of propranolol and on the 4th and 8th days of the experiment (Table 5).

RESULTS

One can see from Table 1 that the average T_3 levels are significantly lower and the average rT_3 levels higher in a bigger group of old subjects than in the control group, but the difference is not always significant in smaller groups (Tables 2–5). The T_3 levels of old subjects were much more frequently under the normal range of the RIA used (less than 1.23 nmole/l) than that of the control group (32.3% and 6.3% respectively). We neither observed a significant age-related difference between the average T_4 and TSH levels of the groups, nor when comparing the average TSH levels of old persons having T_3 less than 1.23 nmole/l with the values of other old subjects (Table 1). These latter findings ignore the possibility that the observed T_3 decrease is the consequence of inhomogenity of the old age group, i.e. the presence of cases of latent hypothyroidism.

No age-related difference was obtained in the response of T_4 and T_3 to TSH (Table 2). Though the absolute T_3 levels measured in old people were a little lower than in the control, the increase of T_3 4 h after TSH administration (ΔT_3) was very similar to the control (the average increase S.E.M. was 0.67± 0.18 nmole/l in the old age group, and 0.47 ± 0.14 nmole/l in the control). The T_3 increase 4 h after TSH administration in 5 old subjects with an initial T_3 level of less than 1.23 nmole/l, was 0.72 ± 0.27 nmole/l, not significantly different from the average values of the whole group.

During the repeated oral TRH tests, parallel to the increase of TSH, we also examined the consecutive T_3 elevation in the different age groups. One can see from Table 3 that on the first and second day in both groups a similar increase in TSH and a similar consecutive T_3 elevation could be observed. On the third and fourth day neither a TSH increase nor a T_3 elevation could be observed in the old age group, while in the juvenile group a TSH response was still visible on the third day, and the T_3 did not decrease even on the fourth day. A significant and similar T_4 increase could be detected in both groups on the fourth day when compared to the initial values (average ΔT_4 in old age was 15.44 ± 4.5 nmole/l; in the control: 20.12 ± 6.88 nmole/l).

As Table 4 demonstrates, in old subjects the T_3 administration did not raise the blood T_3 levels: all three values were significantly lower when compared with the controls. In spite of this, the second TSH response to TRH was significantly lower.

Vol. 16 1981

TABLE 5. EFFECT OF PROPRANOLOL ON THE PLASMA T_4, T_3 AND rT_3 LEVELS IN DIFFERENT AGES

	T_4 (nmole/1)		T_3 (nmole/l)		rT_3 (nmole/l)	
	Aged 70–90 yr S.E.M. (n = 18)	Aged 20–40 yr S.E.M. (n = 12)	Aged 70–90 yr S.E.M. (n = 18)	Aged 20–40 yr S.E.M. (n = 12)	Aged 70–90 yr S.E.M. (n = 18)	Aged 20–40 yr S.E.M. (n = 12)
Before propranolol	118.26 ± 3.02	117.31 ± 4.61	1.89 ± 0.11	2.00 ± 0.15	0.39 ± 0.02	0.35 ± 0.02
4th day of administration	128.76 ± 10.8	123.33 ± 8.88	1.75 ± 0.10	1.98 ± 0.13	0.46 ± 0.02*	0.42 ± 0.03*
8th day of administration	133.46 ± 11.8	128.81 ± 7.89	1.40 ± 0.08***	2.18 ± 0.22	0.43 ± 0.02	0.49 ± 0.03**

Compared to the initial value:
*$p < 0.02$;
**$p < 0.01$;
***$p < 0.001$.

In the dose used, the administered propranolol diminished only the blood T_3 of the old subjects (Table 5). No T_4 changes could be observed, while rT_3 values were increased in both groups. No negative correlation of T_3 and rT_3 was noticeable in the old age group. In the old subjects we observed an rT_3 elevation on the 4th day of propranolol administration, while a significant T_3 decrease manifested itself only on the 8th day.

DISCUSSION

As possible reasons for the detected decrease in blood T_3 levels in old people, one can take in account: a diminution of the T_3 production in the thyroid gland; a decrease of peripheral T_3 generation from the T_4 monodeiodination; an acceleration in the T_3 metabolic clearance; a change of the T_3 distribution space; decrease of the plasma T_3 binding capacity.

Because of the similar T_4 and T_3 increases 4 h after TSH, and the similar TSH and consecutive T_3 elevation on the 1st and 2nd days of the oral TRH administration in both age groups, we conclude that the T_3 response of the thyroid gland to exogenous and endogenous TSH is not diminished in old age. However, in our investigations the so-called 'blunting' of the pituitary's TSH response to repeated TRH administration (Staub *et al.*, 1978) manifested itself earlier in the old age group than in the control, a sign of decreased TSH secretion. According to data in the literature concerned, no senile involution of the thyrotroph cells could be detected by immunohistochemical research methods carried out on the pituitary gland (Ryan *et al.*, 1979). It is known that in healthy people even small doses of T_3 significantly suppress the TSH response to TRH (Snyder and Utiger, 1972). The same can be observed, according to our data, in old people also at lower T_3 levels, and this indicates a stronger suppressive effect of T_3. We therefore think that because of this stronger suppressive effect of T_3 the pituitary TSH secretion is diminished in old age, and this results in a decreased production of T_3 by the thyroid gland.

Since it is well known that the majority of the T_3 pool is supplemented by T_3 generation from the monodeiodination of T_4 (Chopra, 1976; Glöbel *et al.*, 1975), most authors explain the decrease of blood T_3 levels in old age by a shift of peripheral T_4 monodeiodination from T_3 to rT_3 (Faber *et al.*, 1976; Rudorff *et al.*, 1976; Vosberg *et al.*, 1976; Burrows *et al.*, 1977).

Propranolol acts on the blood T_3 levels initially by inhibiting the T_4 monodeiodination, while the induced rT_3 elevation is said to be the result of the reduction of its metabolic clearance (Lumholtz *et al.*, 1979). The latter was observed in both groups, while the diminution of T_3 was found only in old subjects. This may suggest that the T_4–T_3 monodeiodination is altered in old age. The rT_3 elevation was prior to T_3 decrease, as Faber *et al.* (1980) observed. Since, according to data obtained from the related literature (Hüfner and Grussendorf, 1978), rT_3 was found to be a potent inhibitor of T_4–T_3 conversion, the detected rT_3 elevation in old age could be responsible for the supposed diminution of peripheral T_3 generation.

If we do not presume a decrease of T_3 generation from T_4 in old subjects, it would not be clear why, in the control group, a persistent T_3 elevation, without any endogenous TSH stimulus, could be seen on the fourth day of oral TRH administration, while in the case of old people, simultaneously with the elimination of the TSH stimulus on the third day, the T_3 quickly decreased (Table 3). An acceleration of the metabolic clearance of T_3 in old age (another possible explanation for this phenomenon) could not be detected either in humans nor in experimental investigations done by other authors (Rudorff *et al.*, 1976; Frolkis and Valueva, 1978).

Some scientists suggest that in old age, in cases of low initial T_3 levels the response to TSH is greater than in normal subjects (Burrows *et al.*, 1977) and that, parallel with the diminution of the peripheral T_3 generation, there is a compensatory T_3 hyperproduction in the thyroid gland (Wenzel and Horn, 1976). Our data are contradictory to these findings (there was no greater T_3 increase in old subjects with initial T_3 less than 1.23 nmole/l).

The T_3 distribution space and TBG concentration were not measured in our research, but the former was found by others to be unaltered (Rudorff *et al.*, 1976) and the second was found to be significantly higher in old age (Hesch *et al.*, 1976).

By summarising our data we conclude that in old age both the production of T_3 by the thyroid and its peripheral generation from T_4 are decreased, resulting in a diminution of the blood T_3 levels. Decrease of T_3 production by the thyroid is not only connected with senile involution of the gland or with a loss of sensitivity to TSH, but also with a diminished TSH stimulus. As a sign of decreased secretion, the pituitary TSH content was more readily exhaustible by TRH in old age, and this may be, as we believe, the effect of a more pronounced inhibitory effect of T_3 on the TSH synthesis. Decrease of the peripheral T_3 production may be the result of the inhibitory effect of the elevated rT_3 on the T_4 monodeiodination in old people.

Vol. 16 1981

SUMMARY

The blood T_3 levels were lower and the rT_3 levels higher in 70–90-year-old healthy subjects than in 20–40-year-old people. Similar T_4 and T_3 elevations could be seen after exogenous and endogenous (TRH induced) TSH stimulus in both groups. The pituitary TSH content in old age was sooner exhausted by repeated oral TRH administration. A persistent T_3 elevation after endogenous TSH stimulus could be detected only in the control group, probably as a sign of peripheral T_3 generation. Even small quantities of T_3 significantly suppressed the TRH-induced TSH response in the old age group investigated. Propranolol, at the dosage used, inhibited T_4–T_3 conversion only in the old subjects, while the rT_3 increased in both groups. These findings suggest that a more pronounced inhibitory effect of T_3 on the pituitary TSH release is responsible for the decrease of TSH secretion and consecutive diminution of T_3 secretion from the thyroid in old age. The peripheral generation of T_3 seems to be inhibited too, probably by the elevated rT_3 levels.

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Vol
16
198

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ESTIMATION OF THE TURNOVER RATES OF ^{3}H-URIDINE IN MOUSE SKELETAL TISSUES DURING AGING*†

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16
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INTRODUCTION

THE AGE-ASSOCIATED changes in the morphologic and functional integrity of osteogenic and chondrogenic cells have been recently reviewed by Tonna (1977). Among the many alterations reported have been a marked age-decline in cell proliferation as revealed by the utilization of tritiated thymidine. These changes are accompanied by a comparable reduction in ribonucleic acid (RNA) biosynthesis demonstrated by using labeled uridine as a marker (Singh and Tonna, 1974; Tonna and Singh, 1976) and also in protein synthesis as demonstrated by the utilization of labeled glycine, histidine and proline (Tonna, 1971*a*, 1977; Tonna and Pavelec, 1971). These studies have revealed that cells which elaborate the same matrix and exhibit similar function may show significant regional variations in activity depending upon their location. Apparently, differences in cell metabolism may be due to factors such as cell type (chondroblast versus osteoblast) or may reflect regional differences in functional demands placed upon the same cell type; e.g. metaphyseal endosteal osteoblasts show consistently higher proliferative rates and metabolic activity when compared to disphyseal periosteal osteoblasts. Similarly, epiphyseal chondrogenic cells are more active than the articular cartilage cells (see Tonna, 1977 for review). Indeed, with increasing age the cellular metabolic needs change from demands for growth in younger animals to the minimal values necessary for maintenance of cells and tissues in older animals. We have previously reported the uptake and utilization of ^{3}H-uridine by bone and cartilage cells of young and old mice (Singh and Tonna, 1973, 1974; Tonna and Singh, 1973, 1976). In the present study, data on the utilization of ^{3}H-uridine by osteogenic and chondrogenic cells of aging mice was further analyzed to estimate the biological half-life of the radiotracer and to compare turnover times with age.

MATERIALS AND METHODS

One hundred and forty-four BNL inbred strain of Swiss albino mice were used. The mice were divided into four groups of 36 each at ages 5, 26, 52 and 78 weeks, and maintained on Rockland Mouse pellets and water *ad libitum*. Each mouse was given a subcutaneous injection of 5 μCi ^{3}H-uridine (specific activity 21 Ci/mM)/g body weight. From each age group, 36 mice were sacrificed in groups of three at 15, 30 min; 1, 4, 8, 16 h; 1, 2, 4, 7, 14 and 30 days post-injection. Intact femora were removed, decalcified and routinely processed histologically. Autoradiographs were prepared from 5 μm paraffin-embedded sagittal sections using Kodak NTB-3 liquid emulsion and were stored at 4°C in a cold dry atmosphere for 36 days. The preparations were developed and fixed following standard procedures and stained with hematoxylin.

Osteogenic and chondrogenic cells at both the anterior and posterior aspects of the femur were evaluated as follows: (a) diaphyseal periosteal osteoblasts, one to two cell layers deep; (b) metaphyseal endosteal osteoblasts; (c) articular; and (d) epiphyseal cartilage cells (hypertrophic and proliferative zones only). The number of reduced silver grains were counted over the nucleus and cytoplasm of the four cell types. A total of 2400 labeled cells

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†Uridine-5-^{3}H was obtained from Schwarz Bioresearch, Orangeburg, New York.

(600 cells per cell type) were grain-counted for each time period. Cells with overlapping cytoplasmic or nuclear outlines were not included in the analysis. Background correction of grain counts was made for each slide. The data were plotted on semilogarithmic paper. Graphical analyses were applied to the data according to methods previously reported for the utilization of ^{3}H-amino acids (Tonna, 1971*a*; Tonna, Cronkite and Pavelec, 1963). From these analyses turnover rates of ^{3}H-uridine expressed as half-times were calculated.

RESULTS

Autoradiographic grains were seen over both cartilage and bone 15 min after ^{3}H-uridine administration, the earliest observation period. In all four cell compartments, nuclear uptake of ^{3}H-uridine showed peak values at about 1 h after injection in the 5-week-old mice. With increasing age this peak diminished, broadened, and shifted to 8–16 h after ^{3}H-uridine injection. Subsequently, the administered RNA precursor appeared in the cytoplasm. Hence, cytoplasmic activity occurred chronologically later than in the nucleus. Grain counts over the cytoplasm diminished with age and peak values flattened and broadened from approximately 1 day in 5-week-old mice to about 4 days after ^{3}H-uridine administration in the 78-week-old animals. At each age investigated, epiphyseal cartilage showed significantly greater activity as compared to articular chondrogenic cells. Similarly endosteal osteoblasts were metabolically more active than periosteal osteogenic cells.

Tables 1 and 2 show the turnover half-times for ^{3}H-uridine calculated from graphical analyses of average grain counts over the nucleus and cytoplasm of each of the four (two cartilage, two bone) cell compartments. Semilogarithmic plotting of average grain count data revealed a biphasic character when nuclear data were examined but a monophasic nature to the cytoplasmic values. The fast or early component of the nuclear data is expressed in hours and ranged from 3.5 h in endosteal osteoblasts from young mice to 9.8 h in epiphyseal cartilage cells of 78-week-old animals. Endosteal osteoblasts showed a lower value of this component and thus a faster turnover of the radiotracer than the periosteal osteoblasts. Similarly, epiphyseal cartilage cells showed greater metabolic activity than articular cartilage cells. With age, over every cell compartment except the articular cells, there was almost a doubling in time of this fast component. The second (slower) nuclear component was estimated in days. It showed a half-life ranging from 6.5 days in periosteal cells of 5-week-old animals to 13 days in epiphyseal cells of old animals (78 weeks). With age, there was an increase in the estimated half-life, indicating a decrease in metabolic activity. Epiphyseal cell nuclei showed faster metabolic activity than articular cartilage cells while endosteal osteoblasts were more active than the periosteal cells. Estimation of the biological half-life of ^{3}H-uridine over the cytoplasm was monophasic ranging from 6.2 days in endosteal osteoblasts at 5 weeks of age to 11.3 days in

TABLE 1. APPROXIMATE HALF-TIMES ($t_{\frac{1}{2}}$) OF THE FAST (HOURS) AND SLOW (DAYS) CURVE COMPONENTS FROM AUTORADIOGRAPHIC GRAIN-COUNT DATA OVER OSTEOGENIC CELLS AFTER ^{3}H-URIDINE ADMINISTRATION

Age (week)	Periosteal osteoblasts			Endosteal osteoblasts		
	Nucleus		Cytoplasm	Nucleus		Cytoplasm
	I (h)	II (day)	(day)	I (h)	II (day)	(day)
5	4.0	6.5	9.5	3.5	7.0	6.2
26	4.5	9.0	8.3	3.5	7.4	6.5
52	7.5	11.25	7.7	6.5	8.3	8.5
78	9.0	10.75	10.5	8.0	12.5	8.25

Each value is derived from grain-counts over 600 labeled cells.

TABLE 2. APPROXIMATE HALF-TIMES ($t_{\frac{1}{2}}$) OF THE FAST (HOURS) AND SLOW (DAYS) CURVE COMPONENTS FROM AUTORADIOGRAPHIC GRAIN-COUNT OVER CHONDROGENIC CELLS AFTER ^{3}H-URIDINE ADMINISTRATION

Age (week)	Articular cartilage cells			Epiphyseal cartilage cells		
	Nucleus		Cytoplasm	Nucleus		Cytoplasm
	I (h)	II (day)	(day)	I (h)	II (day)	(day)
5	6.3	7.0	10.0	4.0	7.3	8.0
26	6.3	7.3	10.5	4.2	7.5	8.0
52	8.8	9.0	10.8	9.3	9.5	10.8
78	8.7	8.8	11.3	9.8	13.0	10.8

Each value is derived from grain-counts over 600 labeled cells.

articular cartilage cells of 78-week-old mice. An age associated increase in the doubling time was seen although this increase was more moderate than over the nucleus.

DISCUSSION

By biochemical extraction procedures, Burchard and Mandell (1958) demonstrated a high RNA to DNA ratio in bone indicating a relatively higher content of RNA in osteoblasts. The appearance of RNAse-extractable material, suggesting the passage of template RNA into cytoplasmic sites of protein synthesis occurred chronologically later in the cytoplasm of osteoblasts (Burchard *et al.*, 1959). In the present study, both bone and cartilage cell compartments showed nuclear uptake of ^{3}H-uridine to be initially significant; at 8–16 h the increasing cytoplasmic label equalled that observed over the nucleus. Subsequently the diminishing nuclear counts were superseded by the cytoplasmic counts by about 2 days. At 1 month both nuclear and cytoplasmic counts attained similar levels.

Both *in vitro* (Harris and Watts, 1962; Watts, 1964) and *in vivo* studies (Owen, 1967; Tseng *et al.*, 1971) have indicated that RNA precursor pools are small and that when injected, labeled uridine clears the circulation rapidly. One half of the injected dose has been estimated to clear the circulation within 6 min (Tseng *et al.*, 1971), an additional 4 min later, only 4 per cent of the injected dose can be detected (Owen, 1967). The levels of nucleic acids and perhaps their turnover rates may be hormonally dependent and also appear to respond to dietary changes (Mills, 1971). RNA levels in pancreas were reported to be higher in rats fed high soybean diets (Konijn and Guggenheim, 1967; Bredenkamp and Luck, 1969). On the other hand, caffeine appeared to decrease DNA synthesis with no effect on RNA synthesis (Mitznegg *et al.*, 1971). The duration of DNA synthesis in mammary gland alveolar cells was reported to be accelerated under the influence of estrogenic hormones (Bresciani, 1965).

Although some incorporation of ^{3}H-uridine into DNA is known to occur (Jordan and Wilson, 1970; Schaer *et al.*, 1969; Singh and Tonna, 1976), the high specificity of this radiotracer for RNA particularly in tissues with low proliferative activity such as bone and cartilage has been demonstrated (Singh and Tonna, 1976). The uptake of labeled uridine has been widely used to assess RNA content, synthesis or turnover rate in a variety of tissues (Harris and Watts, 1962; Kanungo *et al.*, 1970; Loeb *et al.*, 1965, Owen, 1967; Sasaki and Baserga, 1970; Singh and Tonna, 1973, 1974; Srivastava, 1967; Tonna and Singh, 1973, 1976). The pattern of ^{3}H-uridine labeling of osteogenic and chondrogenic cells was similar to that reported for other mammalian cells. The age-associated decline in the magnitude of the label and the flattening and shifting of peak activity towards later time periods appear to reflect diminished physiological demands and have been discussed

Vol. 16 1981

earlier (Singh and Tonna, 1974; Tonna and Singh, 1976). These changes mirrored similar findings in the synthesis of matrix proteins as revealed by the uptake of labeled glycine, histidine and proline by these cell compartments (Tonna, 1965*a, b*; 1977). RNA/DNA ratio in the fetal brain was reported to increase during prenatal development (Howard *et al.*, 1969), whereas the absolute amount of RNA in the aortic wall decreased during gestation along with an increase in collagen (Berry *et al.*, 1972). RNA content of lizard skin has been reported to decrease with age (Mishra and Patnaik, 1974).

Turnover rates of RNA after injection of a labeled precursor have been estimated by a number of investigators (Amano *et al.*, 1965; Crippa, 1966; Kramer *et al.*, 1973; Palmquist *et al.*, 1971). The present study is the first to report RNA turnover rates by bone and cartilage cells. RNA synthesis *in vitro* was reported to be lowest during pre-DNA-synthetic phase (G_1), increasing rapidly during the DNA-synthetic phase (S) and remaining constant during post-DNA-synthetic phase (G_2) (Crippa, 1966; Konrad, 1963).

In vivo studies of liver and pancreas following injection with labeled cytidine indicated that the nuclear kinetic data could be interpreted as being composed of three different but successive linear portions, each linear component representing a distinct RNA pool with turnover rates estimated to be 4h, 14.5–18.2 h and 5.1–7.4 days respectively; however, cytoplasmic RNA turnover ranged from 10.3 to 13.2 days (Amano *et al.*, 1965). A species difference may exist in the turnover rate of RNA; Palmquist *et al.*, (1971) estimated RNA turnover time in the lactating mammary gland to be 7.6 days in the rat but 14.6 days in the cow. Drugs may effect the turnover rate; e.g. phenobarbital decreased the turnover rate of RNA in cytoplasmic ribosomes from 4.9 to 5.9 days (Vacha and Seifert, 1975).

Amano *et al.* (1965) identified three fractions (pools) within the nuclear RNA. It is generally accepted that of the three main types of RNA, messenger RNA makes up the smallest fraction with a turnover approximating 1 h in mammalian cells (Scherrer *et al.*, 1963), soluble or transfer RNA constituting 10–15 per cent of the cell RNA has a turnover time of about 1 day and ribosomal RNA which forms the main bulk shows a doubling time of 1 week or longer (Davidson, 1960). The three pools isolated by Amano *et al.* (1965) appear identifiable with the three major species of RNA. In the present study, only two nuclear pools of RNA were identified whose doubling time were respectively measured in hours and days. These observations can probably be explained by the fact that the technique used in this study can only monitor the label incorporated into the "stable" or "insoluble" component of RNA and not the larger portion incorporated into soluble form which was lost during processing. This may also account for the fact that in the present study, turnover rate of the faster component was estimated to be somewhat slower (3.5–9.8 h), although cytoplasmic turnover time was comparable to published data (Amano *et al.*, 1965). The appearance of RNA in the nucleus is known to occur sequentialiy within the nucleolus, chromosomes, and nuclear sap respectively (Ringborg and Rydlander. 1971). From the data of the present study, it was not possible to isolate and analyse the three nuclear pools.

As revealed by a faster doubling time of RNA, metaphyseal osteoblasts indicated a high metabolic activity than periosteal cells. Similarly, epiphyseal cartilage cells were more active than articular cells. These findings are consistent with published reports of the utilization of labeled glycine, histidine, proline, thymidine and uridine by these cell compartments (Tonna, 1965*a, b*, 1971*a, b*, 1977; Singh and Tonna, 1973, 1974; Tonna and Singh, 1973, 1976).

An age-associated increase in the turnover times of RNA in both the nucleus and cytoplasm was noted in osteogenic and chondrogenic cells. This decrease in uridine utilization with age reflects a dimunition in the functional demands of growth and development to the levels necessary for maintenance of these tissues. In an earlier study of the utilization of ^{3}H-proline by osteoblasts and chondroblasts, Tonna (1971*b*) demonstrated a biphasic character to the kinetics of autoradiographic grain count data. In addition, an increase was demonstrated in the turnover time of labeled proline by these cells particularly from age 26 to 52 weeks, but not in animals younger than 26 weeks. Animals older than 52 weeks were not examined. Apparently, the demands of growth are intense before 26 weeks. Deleterious aging changes in BNL mice are, therefore, best seen after that age. The findings of the present study are consistent with earlier results (Tonna, 1971*b*).

The age related decline in the RNA-protein synthesizing mechanism was relatively less marked in the articular cartilage cells as compared to epiphyseal cells and again relatively less severe in periosteal cells as compared to endosteal osteoblasts. These observations are consistent with the fact that the endosteal and epiphyseal cells though initially more active have their most important functional role only during active growth and development whereas the articular and periosteal cells necessarily retain important functional activity in the maintenance of tissues throughout the life-span even during aging. The endosteum retains its activity associated with bone remodeling.

Vol. 16 1981

The increase in turnover rate of cytoplasmic RNA is consistent with recent electron microscopic evidence of an age-associated decline in the number of ribosomes and in rough endoplasmic reticulum (Tonna, 1975). It has also been recently suggested that synthesis of ribosomal proteins in the cytoplasm may be independent of nuclear synthesis of ribosomal RNA (Craig and Perry, 1971). Gibas and Harman (1970) failed to find any changes in RNA polymerase activity in nuclei isolated from brain and liver of aging rats.

The aging and maintenance of skeletal tissues is a complex phenomenon presenting deleterious enzymatic age-changes superimposed on related hormonal modifications. Adelman *et al.* (1972) has demonstrated a progressive age associated increase in the time required to initiate many enzyme systems. During development RNA synthesis has been shown to respond more to changes in enzyme systems than to DNA template availability (Marchok and Wolff, 1968). Age changes have been shown to be accelerated with a breakdown in the integrity and maintenance of articular and epiphyseal cartilage during thyroid deficiency (Silberberg and Silberberg, 1954; Silberberg and Hasler, 1968). Hydrocortisone was reported to depress matrix protein synthesis by cartilage cells (Mankin and Conger, 1966) but apparently stimulated collagen synthesis and extrusion by fibroblasts *in vitro* (Macieira-Coelho and Loria, 1976). The cumulative effects of the associated hormonal and deleterious age changes have important implications in tissue maintenance and in the capacity for wound healing during aging.

SUMMARY

Age associated changes in turnover rates of ^{3}H-uridine by osteogenic and chondrogenic cells was autoradiographically investigated. One hundred and forty-four BNL mice, from 5 to 78 weeks of age, were injected with 5 μ Ci of ^{3}H-uridine/g body weight and were killed from 15 min to 30 days later. Autoradiographs were prepared from femora and grain counts were obtained over the nucleus and cytoplasm of labeled periosteal and endosteal osteoblasts as well as articular and epiphyseal cartilage cells. Nuclear turnover of ^{3}H-uridine demonstrated a biphasic character: a rapidly metabolizing fraction with a

biological half-life ranging from 3.5 h in endosteal osteoblasts to 9.8 h in epiphyseal cartilage cells; and a slower metabolizing fraction with half-lives ranging from 6.5 days in periosteal cells to 13.0 days in the epiphyseal cells. Estimated half-lives of ^{3}H-uridine over the cytoplasm ranged from 6.2 days in the endosteum to 11.3 days in the articular cartilage. With increasing age, there was an apparent decrease in total cell activity and in turnover of RNA in all cell compartments. Articular cartilage was the least metabolically active compartment in the young animal, but suffered the least relative decrease with age in ^{3}H-uridine metabolic activity. Epiphyseal cartilage cells showed the greatest age-decline. Age changes in the biological half-life of ^{3}H-uridine reflect concomitant decrease in physiological needs.

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Vol.
16
1981

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LONGEVITY IN SPONTANEOUSLY HYPERTENSIVE MICE*

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16
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INTRODUCTION

IT HAS been known for some time from insurance surveys that in human populations untreated blood pressures in excess of 140/90 are associated with significant life shortening and that pressure below 110/70 are optimum with regard to longevity (Lew, 1973). Life span has not been studied in many of the existing animal models for hypertension, nor in the few models for hypotension. If the models mimic hypertension in man one would expect the same association between life span and blood pressure in the model as in human populations. This study examines longevity in mice of a line developed by selective genetic breeding for elevated blood pressure. About half the mice in this colony have systolic blood pressures in excess of 150 mm Hg. Life span was also studied in a second line selectively bred for low blood pressure in which many of the mice routinely have pressures below 75 mm Hg systolic.

MATERIALS AND METHODS

The mice were samples from the BPI lines genetically selected for high and low systolic blood pressure and from a random-mated control line. The original base population for the two-way selection was produced by an eight-way cross of inbred strains (LP/J, SJL/J, BALB/cJ, C57BL/6J, 129/J, CBA/J, RF/J, and BDP/J) and selection for extreme blood pressures was practiced in the two closed lines for 18–20 generations. A random-bred control derived from the same base population was maintained throughout the study (Schlager, 1974).

Two longevity studies were undertaken. In the first, breeders from the 18th generation of selection were maintained as mated pairs for their lifetime. Earlier litters were left with the parents until weaning, while later litters were removed during the first week after birth. Lifetime reproduction was recorded for all cages, as was age at death. In the second study, virgin mice were set aside at weaning and observed for their lifetime. In this study only cages containing three like-sex litter mates were used to assure initial equal densities. As animals died they were not replaced so densities varied as the study progressed. Female mice could have been combined to maintain three to-a-cage but non-littermate males will fight if introduced into another male's home cage. Consequently, in order to have a valid comparison between the lifespan of males and females, neither was regrouped to maintain equal densities throughout the study. Data in this study were limited to those animals where death was due to natural causes. A few mice were killed for humane reasons (badly injured in fighting, etc.) and a few disappeared during the three year period of the study. The cages were checked at least weekly and generally two or three times a week. The major differences between the two studies were: (1) The differences in average blood pressure between the High and Low line were greater in the first study since only the more extreme mice are used to propagate the line. (2) The variation of blood pressure was greater within the lines in the second study, although there was no overlap of the Hypertensive (HBP = high blood pressure) and Hypotensives (LBP = low blood pressure) distributions. (3) The randombreds were not included in the first study but were set out in a similar manner as the selected lines in the second study. (4) Litter sizes were recorded as soon after birth as practical in the first study; the mice in the second study were not mated.

The systolic blood pressure (SBP) of all mice was taken when they were 100–150 days of age. The method used involved an occluding cuff on the tail and electronic/pneumatic detection of pulse distal to the cuff while the mice were restrained but not anesthetized (NARCO BIOSYSTEMS Physiograph; method described by Schlager, 1974). Five individual blood pressures (BP) were taken on each of three separate days about a week apart.

Although genetic selection was practiced on systolic blood pressure, diastolic pressures changed proportionally. In the 14th generation of selection diastolic blood pressures measured directly from the femoral artery under ether anesthetic was 99 ± 5 mm Hg in the HBP and 59 ± 8 mm Hg in the LBP.

*This study was supported in part by the General Research Fund of the University of Kansas.

The mice were housed in stainless steel cages 11 in × 5 in × 6 in and fed Purina Laboratory Chow and provided with water *ad libitum*. Constant light cycle of 12 h and temperatures of 70–80°F were maintained throughout both studies.

Comparisons between LBP and HBP in the first study were made by a *t*-test. In the second study a one-way analysis of variance followed by a studentized multiple comparison of means was used.

RESULTS

Bred mice

The average lifespan was higher in the LBP for both sexes; in each case about one half of the LBP mice lived longer than all of the HBP (Figs. 1 and 2, and Table 1). For females this difference is striking as the entire survival curve of the HBP is below LBP. In males there is some early crossing of the two survival curves. The survival curve also shows a more precipitious drop in the HBP than the LBP in both sexes, although somewhat more pronounced in the males. Within the lines, there was a positive correlation between age at death and systolic blood pressure in the LBP line but a negative correlation in the HBP line (Table 1). None of these correlations were statistically significant although that within the LBP males approaches $p = 0.05$.

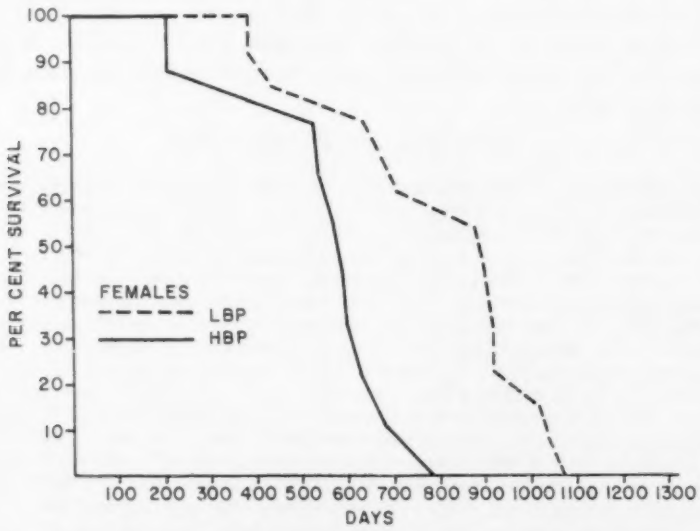

FIG. 1. Survival curves of bred females of the LBP and HBP lines. Mice housed together as a mated pair for life.

The influence of lifetime reproductive history on lifespan was also examined. The mean number of offspring produced by the HBP females was almost twice that of the LBP although the average number of litters was about five in each line. There was a negative association between number born and lifespan and between number of litters and lifespan in the LBP females, and a positive correlation in the HBP females. The correlation coefficient for number of litters and lifespan was statistically significantly different from zero in the HBP females; none of the other correlations were statistically significant.

Unmated mice

The LBP females outlived those of the HBP line by about 200 days (Fig. 3, and Table 2). The LBP males also lived longer than those of the HBP lines, about 300 days longer

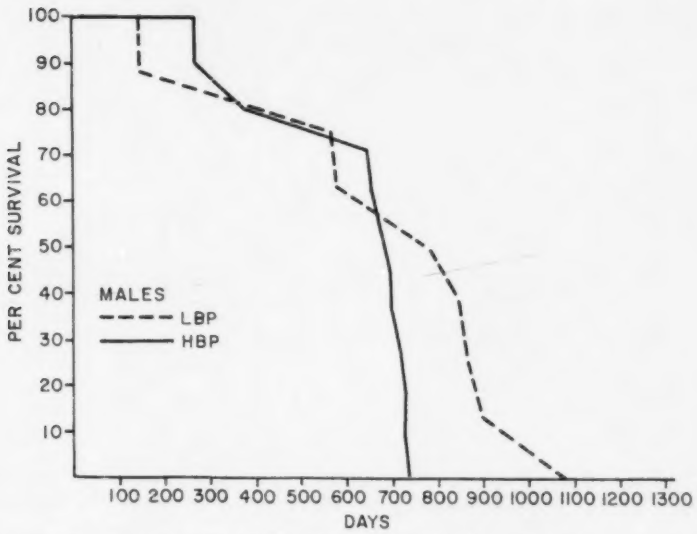

FIG. 2. Survival curves of bred males of the LBP and HBP lines. Mice housed together as a mated pair for life.

TABLE 1. MEANS AND STANDARD ERRORS FOR LIFESPAN, SYSTOLIC BLOOD PRESSURE (SBP) AND REPRODUCTIVE PARAMETERS FOR THE BRED MICE OF THE FIRST STUDY

Line	Sex	Sample size	Age at death (days)	SPB at 100–150 days (mm Hg)	Number of offspring per female	Number of litters per female	Correlations between lifespan and SBP	Number born	Number of litters
HBP	♀	9	571 ± 52*	166 ± 4†	43 ± 7*	5.2 ± 0.6	−0.093	0.562	0.679‡
LBP	♀	13	808 ± 62	76 ± 2	25 ± 4	4.9 ± 0.8	0.128	−0.360	−0.308
HBP	♂	11	628 ± 46	165 ± 4†			−0.220		
LBP	♂	8	719 ± 100	76 ± 3			0.542		

*A significant difference exists between HBP and LBP of the same sex at $p < 0.05$.
†$p < 0.01$.
‡Correlation coefficient significantly different from zero at $p < 0.05$.

(Fig. 4). Both the LBP and randombred had significantly longer lifespans than the HBP in both studies. The correlation between blood pressure and lifespan was negative in both the LBP and HBP. These correlations were not statistically significant.

DISCUSSION

The data support the hypothesis that elevated blood pressure does not favour long life. The average lifespan was higher in the Hypotensive line than in the Hypertensives in both studies in both sexes. Extensive actuary studies in human populations demonstrated that high blood pressure (both hypertension and borderline hypertension) is associated with significant life shortening, while pressures 10–15 mm Hg below average are optimal with regard to longevity (Lew, 1973). For the retired breeders in our study, the LBP mice lived 15 to 40 per cent longer than the HBP. Among the nonbreeders the HBP again had shorter lifespans. However, the extreme blood pressure individual among breeders tended to be at a disadvantage with respect to longevity within both lines, since there was a positive association between blood pressure and lifespan in the LBP but a negative association in the HBP.

Vol. 16 1981

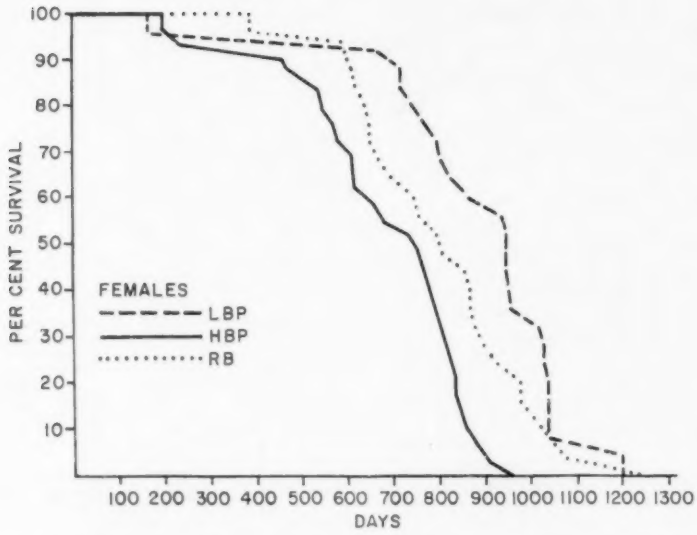

FIG. 3. Survival curves of the unmated females of the LBP, HBP and randombred lines. Initial density was three like-sexed mice per cage.

TABLE 2. MEANS AND STANDARD ERRORS FOR LIFESPAN, SYSTOLIC BLOOD PRESSURE (SPB) OF UNMATED MICE OF THE SECOND STUDY

Line	Sex	Sample size	Age at death (days)	SPB at 100–150 days (mm Hg)	Correlation between lifespan and SBP
HBP	♀♀	29	691 ± 35*	122 ± 4†	−0.18
LBP	♀♀	25	902 ± 42	68 ± 3	−0.16
RANDOM	♀♀	25	814 ± 38		
HBP	♂♂	31	551 ± 38*	131 ± 5†	−0.09
LBP	♂♂	25	853 ± 39	83 ± 3	−0.10
RANDOM	♂♂	24	842 ± 28		

*HBP significantly different from LBP and randombred at $p < 0.05$.
†HBP significantly different from LBP at $p < 0.01$.

Bred females did not have as long a lifespan as the unmated females in either LBP or HBP. Male mice of the HBP lived longer when allowed to mate throughout their lifetime than when unmated. On the other hand unmated LBP males had somewhat longer lifespans than bred LBP males. These comparisons must be made with some caution since these experiments were not designed to be compared to one another and were not run concurrently. Since only mice with extreme blood pressures are used to propagate the lines, the differences between the HBP and LBP were greater in the first study involving bred mice than in the second involving unmated mice. The longer life span of the unmated females of the HBP compared to the bred female may reflect the higher BP of the bred females rather than reproductive stress. Similarly, the longer life span of the unmated LBP compared to bred LBP may be due to the lower BP in the bred mice and the positive association between BP and life span.

There are a number of confounding variables in this study which may contribute to the differences in survival between the HBP and LBP lines. For the bred mice the stress of reproduction could have had an effect on lifespan. On average the LBP female produced 25 offspring in five litters while the HBP produced 43 in about the same number of litters. The smaller litter sizes in the LBP line has been observed for a number of generations and has led to near extinction of the line on at least two occasions. This reduction in litter size has been considered an effect of natural selection where the lowered blood pressure may not suffice to physiologically support larger litters. Virgin female mice tend to have significantly longer lifespans than bred females (Russell, 1966) but not consistenty so (Hrubrant, 1964). The size of litters and longevity appear to be genetically correlated in mouse strains when one omits strains with known specific diseases (e.g., leukemia) and examines only females that have had five or more litters (Roderick and Storer, 1961). There has also been some evidence presented to show that the albino locus may be associated with shorter lifespan, but this has been refuted by other (Goodrick, 1975). Single gene substitutions at the agouti locus also seems to affect survival by nine to fourteen per cent (Hrubrant, 1964). The albino locus was fixed in the HBP line for several generations while tans, brown and dilute alleles are noticeably segregating in the LBP line. It is more likely that the differences in longevity observed in our study are due in large measure to the two-fold difference in blood pressure rather than the reproductive history or pleiotrophic effects of single gene differences for coat color.

Vol. 16 1981

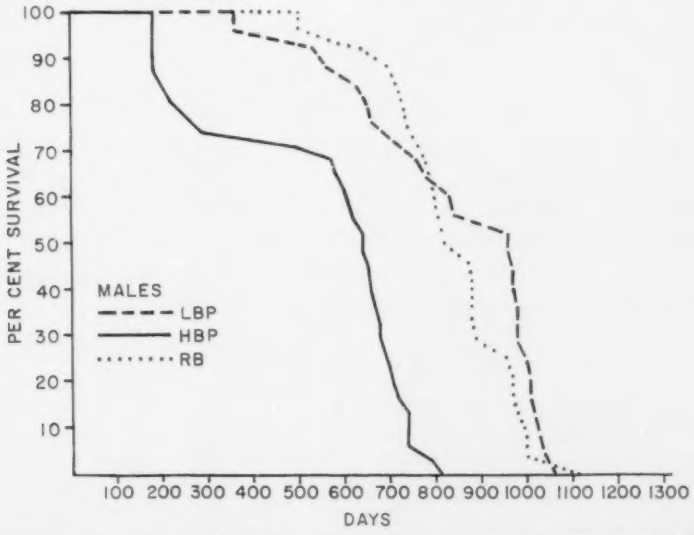

FIG. 4. Survival curves of the unmated males of the LBP, HBP and randombred lines. Initial density was three like-sexed mice per cage.

In rats, Drori and Folman (1969, 1976) have demonstrated that mated males live significantly longer than their unmated littermates although forcing a male rat to run in a revolving steel drum for 2 min a day produced a similar survival curve (Drori and Folman, 1976). Although the experiments reported here were not designed to test a hypothesis of longer life for mated male mice, a comparison of the survival data for the males of the LBP line in the two studies suggest that the mated males did not live

as long as unmated males. However, our nonmated data comes from males caged with two other males while the mated data comes from males caged with one female and their periodic litters. Ebbesen (1972) has shown that unmated single male mice in a cage live considerably longer than males housed with five or six other males.

Our findings in the spontaneously hypertensive mouse parallel those of the spontaneously hypertensive rat (SHR) which also has a significantly shorter lifespan when compared to Wistar controls. Nagaoka *et al.* (1972) found a significant decrease in longevity of the SHR when compared to WK controls. About 60 per cent of the male SHR and 30 per cent of the female SHR died before any of the Control rats. The blood pressure levels in the spontaneously hypertensive mouse are not as elevated as those of the SHR, but it is evident that even mild elevation of blood pressure can lead to life shortening.

Dupont *et al.* (1975) reported significantly shorter life span in rats selected for high blood pressure of the Lyon strain compared to normtensive and hypotensive rats. All male hypertensive rats in this study died by age 32 months at which time approximately 80 per cent of the female hypertensive rats were dead. Among females of the other two lines, fewer than 25 per cent died by 32 months. Lifespan was shorter in the males where approximately 70 per cent of normotensives and 50 per cent of the hypotensives died by 32 months.

In three independent studies of potential animal models for human hypertension, animals with elevated blood pressures had significantly shorter lifespans. In the Lyon hypotensive rat and the LBP mouse the effect of low blood pressure is less clear, but appears to be associated with somewhat increased lifespan.

SUMMARY

Longevity was investigated in three lines of mice with genetically selected differences in systolic blood pressure. Lifespan was shortest in the line with elevated blood pressure and significantly longer in the normotensive and hypotensive lines. In unmated animals this difference in lifespan between the low blood pressure line and high blood pressure line was 200–300 days. The difference in the bred mice was somewhat less but was confounded by differences in reproductive capacity.

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METHYLATION OF CHROMOSOMAL PROTEINS AND DNA OF RAT BRAIN AND ITS MODULATION BY ESTRADIOL AND CALCIUM DURING AGING

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Vol. 16 1981

INTRODUCTION

POSTSYNTHETIC MODIFICATIONS of chromosomal proteins such as methylation, acetylation and phosphorylation are believed to be involved in the organization and function of chromatin (Allfrey, 1971; Kleinsmith, 1975; Barrett *et al.*, 1978). Methylation of histones raises the PK of ε-NH_2 group resulting in an increase in the basicity of histones (Gershey *et al.*, 1969). Incorporation of methyl groups into certain histones has also been implicated in the process of development (Borun *et al.*, 1972). The significance of methylation of non-histone chromosomal (NHC) proteins is not yet completely understood. Shortly after the synthesis of DNA, cytosine is methylated to 5-methyl cytosine (Burdon and Adams, 1979). This may influence the binding of histones to DNA and thereby cause a change in the conformation of chromatin. Methylated cytosines also prevent cleavage of DNA by specific endonucleases (Bird and Southern, 1978). Methylation of DNA is also believed to be involved in the control of differentiation (Holliday and Pugh, 1975).

Steroid hormones regulate the number of transcription sites available in DNA (Teng and Hamilton, 1969). They also stimulate modifications of chromosomal proteins (Schmidt and Goodman, 1976; Kanungo and Thakur, 1979). This may alter gene expression. Calcium displaces histones and interacts with phosphate groups of DNA (Chang and Carr, 1968). Thus, hormones and cations may modulate gene activity by altering the interaction of chromsomal proteins with DNA. Changes in gene activity may account for the attainment and maintenance of different phases of the life span. The present work was undertaken to determine the age-related methylation of chromosomal proteins and DNA of the brain of the rat to get some insight into the possible changes that may occur in gene function during aging.

MATERIALS AND METHODS

Female albino rats of Wistar strain were used. Immature (2-), adult (15-) and old (84-week) rats were killed by cervical dislocation. The cerebral cortex was excised and cut into slices of approximately 0.4 mm thickness (Takagaki *et al.*, 1959). Methylation of chromosomal proteins was studied according to Sung *et al.* (1977). The tissue slices were preincubated with cycloheximide (2×10^{-4}M) for 30 min to inhibit the incorporation of labelled methionine during protein synthesis. 17β-estradiol (1 μmole) or calcium gluconate (5 μmole) was added to experimental flasks. The control and experimental flasks were set up in duplicate and shaken for 30 min in a water bath maintained at 37°C. Then 20 μCi of (methyl-^{14}C) methionine (50 mCi/mmole) was added to both control and experimental flasks, and shaking was continued for 60 min.

The slices were taken out and washed with cold Krebs–Ringer bicarbonate buffer. They were homogenized in

saline-EDTA and centrifuged to obtain nuclear pellet. Chromatin was purified through sucrose gradient (Bonner *et al.*, 1968). Histones were extracted with 0.4 N H_2SO_4. The dehistonized chromatin was treated with bovine pancreatic DNase I (Sigma) to separate DNA from NHC proteins (Wilson and Spelsberg, 1973). Histone, NHC protein (Lowry *et al.*, 1951) and DNA (Schneider, 1957) contents were determined. The incorporation of ^{14}C methyl groups into each fraction was measured in a Beckman LS-100C liquid scintillation system.

The histone fraction was pelleted with absolute ethanol and analysed electrophoretically in 6.25 M urea-5.4% (v/v) acetic acid (Panyim and Chalkley, 1969). The NHC proteins were analysed electrophoretically in SDS-polyacrylamide gel in SDS-tris-glycine buffer, pH 8.3. The protein bands were sliced and digested with 30% H_2O_2 overnight at 37°C. The radioactivity in each band was counted using triton-toluene scintillation fluid.

RESULTS

Table 1 shows that methylation of histones, particularly of H3 and H4, declines gradually with increasing age of the rat. Estradiol and calcium stimulate methylation of histones in immature and adult rats. Though the hormone stimulates methylation of histone H2B, calcium stimulates that of H3 (Fig. 1). However, these modulatory effects are not observed in old age.

TABLE 1. EFFECTS OF 17β-ESTRADIOL AND CALCIUM ON METHYLATION ($-^{14}CH_3$) OF HISTONES, NHC PROTEINS AND DNA (SPECIFIC ACTIVITY, CPM/μg) OF CEREBRAL CORTEX OF RATS OF THREE AGES

Age (weeks)	Histones			NHC proteins			DNA		
	Control	+ Estradiol	+ Calcium	Control	+ Estradiol	+ Calcium	Control	+ Estradiol	+ Calcium
2	3.25	3.84	3.98	0.38	0.35	0.31	5.92	3.46	9.04
15	2.73	3.02	3.16	0.15	0.19	0.12	8.50	5.85	10.22
84	2.40	2.28	2.33	0.11	0.10	0.12	18.02	18.88	19.91

Tissues were pooled from two to three rats for each incubation. This was done in duplicate. Chromatin was isolated after labelling the tissue with (methyl-^{14}C) methionine. It was fractionated into its constituent proteins and DNA. The concentration and the incorporation of radioactivity were measured as mentioned in the text. The specific activity was calculated as counts per min/μg of protein or DNA. The data given are the mean obtained from four to six rats of each age.

Methylation of NHC proteins is highest in immature rats and decreases thereafter as a function of age. The incorporation of methyl groups into individual NHC proteins varies during different stages of the lifespan. Almost all NHC protein fractions show lower methylation in adult and old rats as compared to those of the immature (Fig. 2). Estradiol stimulates methylation of total NHC proteins of adult rats, but not of the immature and the old. It inhibits incorporation of methyl groups into the first, ninth, 18th, 20th and 30th NHC protein fractions in immature rats. Methylation of several NHC proteins of adult and old rats is influenced by estradiol. On the other hand, calcium has a slight inhibitory effect on the methylation of NHC proteins in immature and adult rats. The extent of inhibition differs for different NHC proteins. However, no significant effect is seen in old rats.

Methylation of chromosomal DNA, unlike that of histones and NHC proteins, increases progressively with age, particularly after the attainment of reproductive maturity (Table 1). It is inhibited by estradiol in immature and adult rats. Calcium, on the other hand, stimulates DNA methylation in rats of all the three ages. However, the degree of stimulation decreases with increasing age.

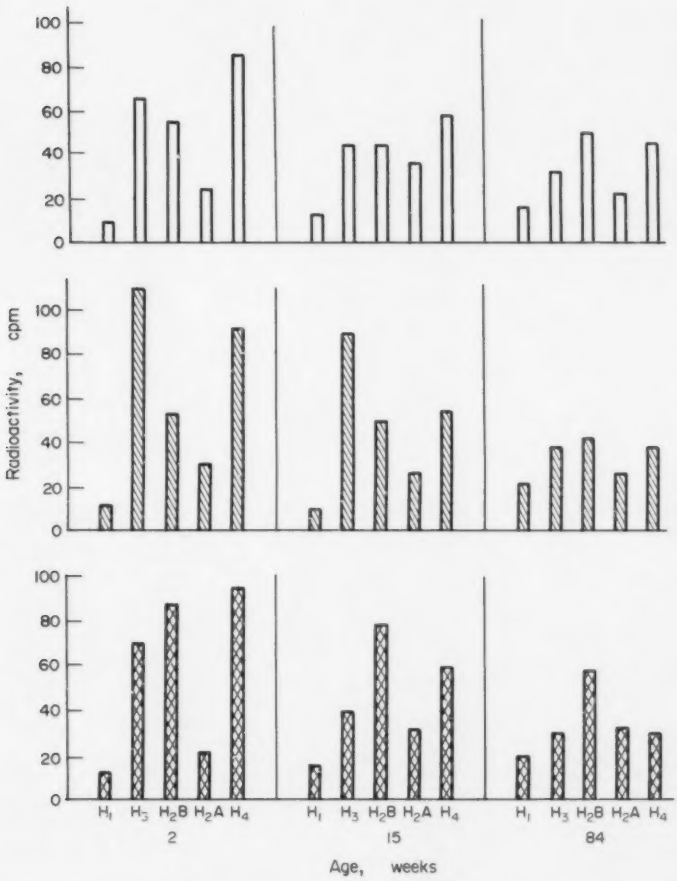

FIG. 1. Effects of calcium (▨) and estradiol (▩) on methylation of individual histones of the cerebral cortex of female rats of different ages.

DISCUSSION

Higher methylation of histones in the immature rat may be the result of rapid neuronal division (Johnson and Kim, 1973). Neuronal division stops after early development, and concomitantly the turnover of histones ceases. So methylation observed after early development may occur at new sites. Since methyl groups on histones do not turnover (Byvoet *et al.*, 1972), the actual number of methylated lysyl residues would increase with age, even though the incorporation of $^{14}CH_3$ groups shows a decrease. Methylation of H3 and H4 which are known to be the most dominant forms of methylated histones is highest in the immature and decreases significantly with age. Methylation of other histones is lower than that of H3 and H4, and also does not change markedly with age. This is of considerable significance since $(H3\text{-}H4)_2$ tetramer defines the length and basic fold of the core DNA (Kornberg, 1974; Camerini-Otero *et al.*, 1976; McGhee and Felsenfeld, 1980). Estradiol enhances transcription (Hamilton *et al.*, 1968) by binding to chromatin together with a cytoplasmic receptor whose level decreases with age (Kanungo *et al.*, 1975).

Vol. 16 1981

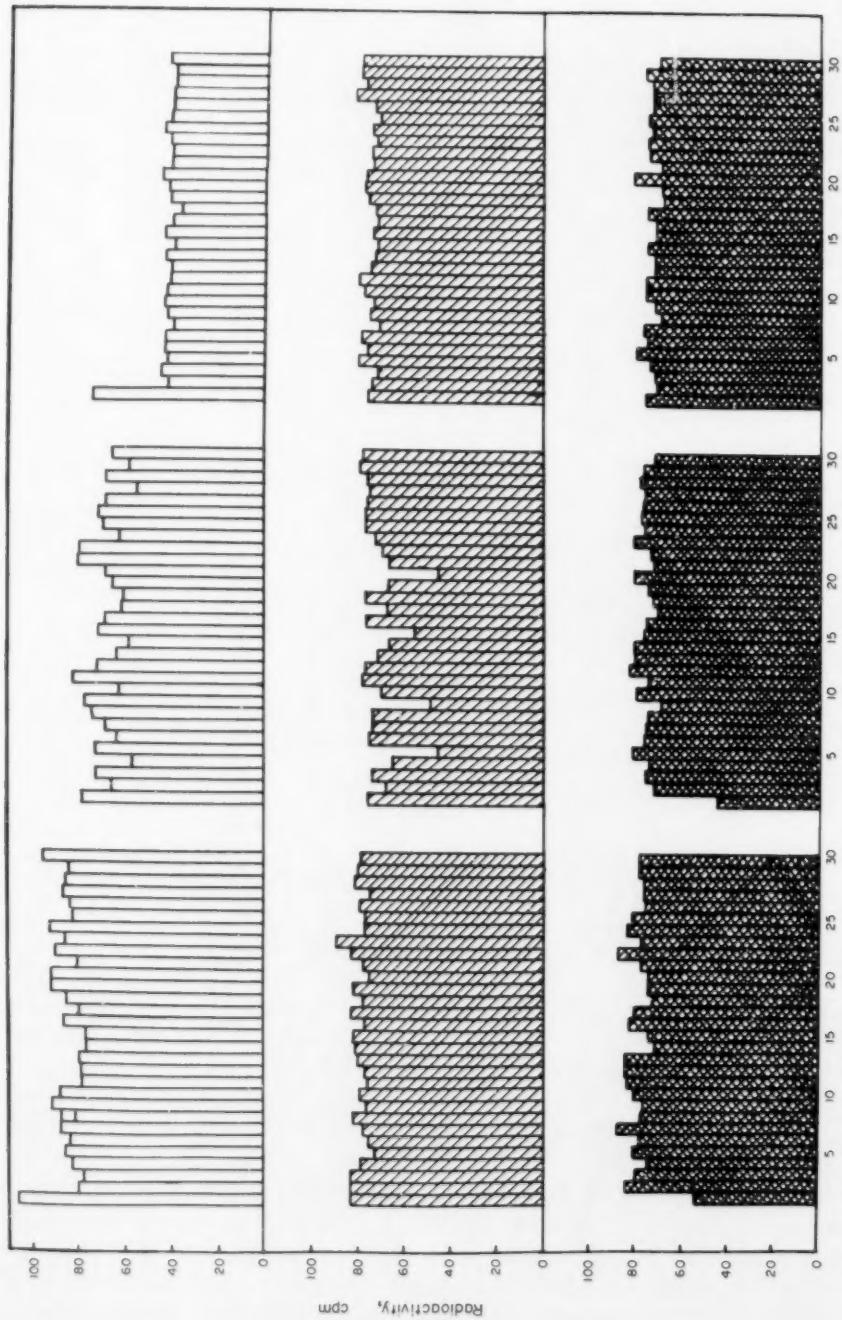

FIG. 2. Effects of calcium (▨) and estradiol (▩) on methylation of individual NHC proteins of the cerebral cortex of female rats of different ages.

However, how it stimulates methylation of histones, and the significance of this effect remains to be elucidated. It is of interest that calcium stimulates methylation of H3, and estradiol that of H2B. Such differential stimulatory effects by two distinct effectors may have different effects on gene expression.

Like histones, methylation of NHC proteins also declines progressively with age. This appears to be due to a general decrease of methylation of all NHC protein fractions. Such a general pattern of change may be due to a decrease in the level of methylase responsible for methylation of NHC proteins. Stimulation of methylation of NHC proteins by estradiol is seen only in the adult. No specific effect on any particular NHC protein is observed. The mechanism by which estradiol and calcium modulate methylation of NHC proteins needs further investigation.

The incorporation of methyl groups into the bases of DNA, particularly into cytosine, may have profound effects on its biological function. It is reported to increase the binding of histones to DNA (von Hahn and Verzar, 1963) and also raises the Tm of chromatin (Vanyushin *et al.*, 1963). This represses the template activity of chromatin. Our data show that methylation of DNA increases significantly with age. Particularly the increase after adulthood is remarkable. Conformational changes in chromatin that may expose and make cytosine more accessible to DNA methyltransferase, and/or an increase in the level of this enzyme may account for the increase in DNA methylation. This may lead to a tighter binding of histones to DNA which may account for a decline in the template activity of chromatin that is seen with increasing age (Kanungo and Thakur, 1979*b*; Thakur and Kanungo, 1980). Estradiol inhibits DNA methylation in immature and adult rats. This may weaken DNA-histone binding and permit transcription at specific loci. Calcium, on the other hand, stimulates DNA methylation. This may strengthen DNA-histone binding and thereby inhibit its template activity.

The present findings show that chromosomal proteins and DNA undergo differential degree of methylation during aging. Furthermore, these modifications are subject to specific modulation by effectors like estradiol and calcium, whose levels vary with age. Hence, the degree of these modulations also changes as a function of age. Such changes may alter the expression of genes and lead to senescence (Kanungo, 1975, 1980).

Vol. 16 1981

SUMMARY

The effects of estradiol and calcium on *in vitro* methylation of histones, NHC proteins and DNA of the cerebral cortex of rats of different ages were studied. Methylation of histones and NHC proteins decreases with age. Estradiol and calcium stimulate methylation of histones in immature and adult rats. Estradiol enhances methylation of NHC proteins only in the adult, whereas calcium inhibits it in immature and adult rats. Methylation of DNA increases with age. It is inhibited by estradiol, but is stimulated by calcium. Such age-dependent changes in methylation of chromosomal proteins and DNA, and their differential modulation by different effectors may destabilize the expression of genes and cause aging.

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CADMIUM: TEMPERATURE-DEPENDENT INCREASE WITH AGE IN *DROSOPHILA*

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Vol.
16
1981

INTRODUCTION

THE JAPANESE Ministry of Health and Welfare established in 1968 that Itai-Itai disease, characterized by skeletal deformities and multiple bone fractures, is caused by chronic cadmium poisoning. Since then there has been considerable interest in the possibility that low level cadmium exposure might be responsible for other degenerative diseases. Based upon the reported age-related accumulation of cadmium in human organs, it seemed of interest to explore the possibility that cadmium accumulation might play an important role in senescence. Elinder *et al.* (1976) have reported an increase with age in hepatic cadmium for human females and a similar increase for males followed by a decline after age 80 for males. Several different studies have demonstrated an age-related increase in renal cadmium up to about 50 years of age followed by a substantial decline in some studies but not in others (Friberg, 1976). In the C57BL/6J laboratory mouse, a similar increase in hepatic and renal cadmium has been observed during senescence (Massie and Aiello, 1979). Until now, the question of whether whole-body cadmium concentration increases with age has not been addressed. We report here that total body cadmium in the fruit fly, *Drosophila melanogaster,* increases with age.

Since the lifespan of *Drosophila* varies inversely with the environmental temperature, as it does for poikilothermic organisms in general, one would expect that if cadmium accumulation were important in the development of senescence that the rate of accumulation of cadmium should vary directly with environmental temperature. We have found this to be true for *Drosophila* maintained at 11, 20, 25 and 30°C.

MATERIALS AND METHODS

Oregon R *Drosophila melanogaster* fruit flies were reared at 25°C from eggs laid on corn meal medium which consisted of 7.6 gm granulated agar (Teklad Test Diets), 173.2 ml Grandma's molasses, 144.7 gm yellow corn meal (Quaker Oats), 29.6 gm nonfat dry milk (Carnation), 70 gm sucrose (Red and White), 7.6 gm dry powdered yeast (Natural Brands) in 1 l of water. Newly emerged flies were placed on corn meal medium in 8 oz. french square culture bottles and maintained at environmental temperatures of 11, 20, 25 and 30°C on a 12:12 hr light–dark cycle. Relative humidity was 70 per cent.

Flies were removed at appropriate time intervals for cadmium determinations. After drying overnight at 88°C, 25 flies were digested in 0.1 ml of Ultrex nitric acid (J. T. Baker Chemical Co.) at room temperature for 1 week or more. Cadmium was determined at 228.8 nm on a Varian 1250 atomic absorption spectrophotometer with carbon-rod atomizer Model 90. The cadmium content of the corn meal medium was 0.012 ng/mg dry wt.

RESULTS

Cadmium concentrations in *Drosophila* eggs were low (0.054 ng/mg dry wt.) but were five times higher than the cadmium content of the corn meal food source. During the larval and pupal stages the cadmium content increased only slightly with values ranging from 0.078 to 0.094 ng/mg dry wt. with no age-related trend.

Adult flies accumulated small amounts of cadmium during the first 2 weeks of life and much larger amounts after 2 weeks, reaching a high of 0.439 ng/mg dry wt. at 8 weeks of age. The overall increase in cadmium content with age was non-linear with the data yielding an apparent exponential curve. We accordingly plotted the logarithm of the cadmium content versus age and obtained a straight line (Fig. 1). From this plot we determined a doubling time of 27.5 days for whole-body cadmium from the egg to the senescent adult stage at 25°C.

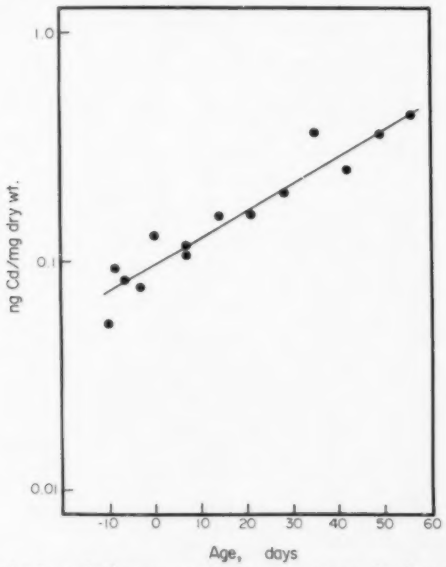

FIG. 1. Logarithm of whole-body cadmium concentration versus age in days for male Oregon R *Drosophila melanogaster* for both developmental and adult stages at 25°C. Negative days represent developmental stages beginning with eggs at −11 days, first and second instar at −8 days, third instar at −6.5 days, early pupa at −5 days and late pupa at −1 day. Cadmium is expressed as ng/mg dry wt.

The well known dependence of lifespan on environmental temperature for *Drosophila* is shown in Fig. 2. In our hands the median lifespan varied from a low of 25 days at 30°C to a high of 152 days at 11°C (Table 1).

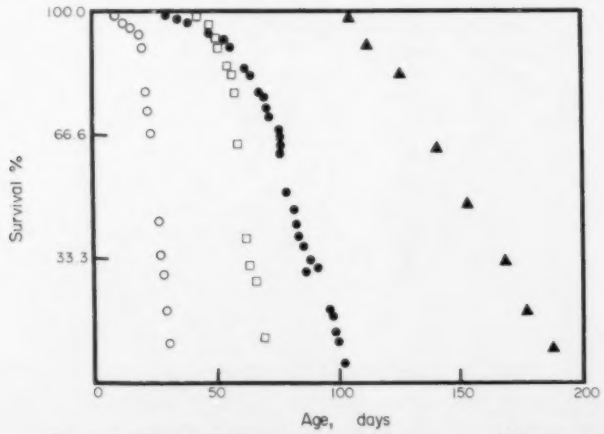

FIG. 2. Survival curves for male Oregon R *Drosophila melanogaster* at different temperatures. 11°C, ▲, 20°C, ●, 25°C, □, and 30°C, ○.

TABLE 1. MEDIAN LIFESPAN AND TIME REQUIRED TO DOUBLE THE WHOLE-BODY CADMIUM CONCENTRATION FOR MALE OREGON R *DROSOPHILA MELANOGASTER* WHEN MAINTAINED FROM EMERGENCE AT VARIOUS TEMPERATURES ON CORN MEAL MEDIUM

Temp, °C	Median lifespan, days	Cd concentration, doubling time, days
11	152	64.0
20	81	35.8
25	62	27.5
30	25	13.5

In order for cadmium to play a role in senescence the rate of accumulation should reflect the rate of aging at different environmental temperatures or the total amount of cadmium might be expected to be greater in flies maintained at higher temperatures. Our results show that the total amounts of cadmium accumulated over a lifetime at different temperatures are about the same (0.4–0.6 ng/mg dry wt.) but the rate of accumulation is temperature-dependent (Fig. 3). The time required for a doubling of whole-body cadmium ranges from a low of 13.5 days at 30°C to a high of 64.0 days at 11°C (Table 1) but the ratio of lifespan to cadmium doubling time is essentially constant at the different environmental temperatures with the exception of a lower value at 30°C. The ratio is 2.4, 2.3, 2.3 and 1.8 at 11, 20, 25 and 30°C, respectively.

Vol. 16 1981

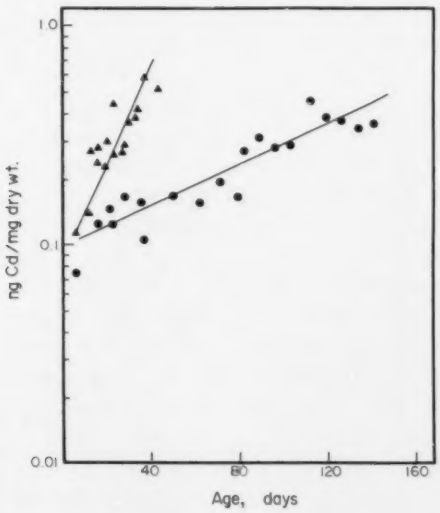

FIG. 3. Logarithm of whole-body cadmium concentration versus age in days for male Oregon R *Drosophila melanogaster* maintained at 11°C, ● and 30°C, ▲. Cadmium is expressed as ng/mg dry wt.

The plot of doubling time versus ambient temperature of maintenance is linear (Fig. 4) and can be used to predict the doubling time for cadmium accumulation at any given temperature.

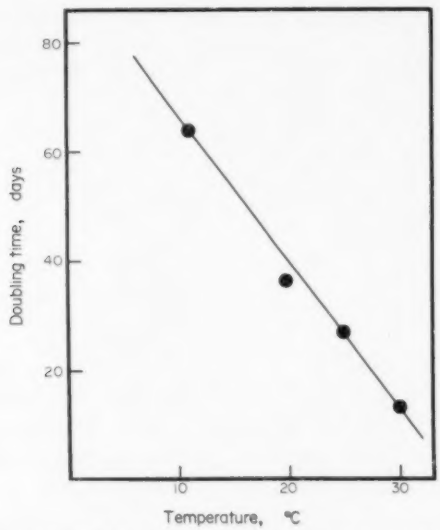

FIG. 4. Time required for doubling of whole-body cadmium content in flies maintained at various temperatures from 11°C to 30°C.

DISCUSSION

The exponential increase in cadmium in *Drosophila* with age parallels the exponentially increasing probability of death which occurs with normal aging (Gompertz, 1825). The data of Elinder *et al.* (1976) for cadmium in kidney cortex also show an exponential increase for humans up to 50 years of age and our previously reported values for cadmium in liver and kidney of C57BL/6J male mice increase exponentially with age (Massie and Aiello, 1979). There appears to be a progressive loss with age of cadmium homeostasis in all species studied so far. This loss could be merely an age-dependent correlation. On the other hand, it could represent a causal factor in senescence.

Based on these observations one might expect that older organisms would have a greater susceptibility to low levels of cadmium. In addition to food, sources of exposure include cadmium-plated metal, nickel-cadmium batteries, some plastics, paint pigments and the burning of fossil fuels. Thus, it seems unlikely that a cadmium-free environment is feasible. Investigations into cadmium homeostasis offer the greatest hope for clarifying the possible role of cadmium in senescence. Certainly, additional analysis for age-related accumulation of cadmium and other toxic metals such as mercury and lead in organisms other than man would be of interest.

In a communication to follow we will report on changes in lifespan produced by cadmium addition and removal.

SUMMARY

The concentration of cadmium increases exponentially with age in adult *Drosophila melanogaster,* reaching a final value of from 0.4 to 0.6 ng/mg dry wt. when flies are maintained on a diet containing low amounts of cadmium. Lowering the environmental temperature reduces both the rate of aging and the rate of cadmium accumulation. The

median lifespan is 152, 81, 62 and 25 days and the corresponding doubling time for cadmium is 64.0, 35.8, 27.5 and 13.5 days for flies maintained at 11, 20, 25 and 30°C, respectively.

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Vol.
16
1981

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VASCULAR DEGENERATIVE CHANGES IN THE UTERINE ARTERIES OF SENESCENT FEMALE GOLDEN HAMSTERS

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Vol.
16
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INTRODUCTION

THE MOST prominent histological feature in aging of the rodent uterus is the accumulation of collagen. There is a progressive increase in connective tissue between the two muscle layers, stroma and around the blood vessels. The amount of collagen increases in the uterus with age (Schaub, 1964; Rahima and Soderwall, 1977), and it has been suggested that increased deposition of collagen might impair uterine function, possibly by interfering with vascularization (Biggers *et al.*, 1962). Larson and Foote (1972) studied the uterine blood flow in young and aged female rabbits and found that the average uterine blood flow rate was higher in young females. In rats, the collagen fibers become more strongly crossed-linked in pregnancy (Schaub, 1964). This change in the collagen, together with the increase in amount of collagen around the blood vessels, could lead to occlusion of the vessels and poor circulation. We therefore investigated this in young and old golden hamsters.

MATERIALS AND METHODS

Golden hamsters (*Mesocricetus auratus*), were maintained on a diet of Purina Laboratory Chow and lettuce greens daily. The vaginal discharge of 10 nulliparous females (3–9 months of age) and 8 multiparous females (15–22 months of age) was checked daily (Orsini, 1961) for the appearance of the post-estrous discharge (day 1) and only animals which exhibited a regular four-day cycle were used. They were then placed with young males (3–6 months of age) of proven fertility on the evening of the pro-estrous period and examined for a vaginal plug or sperm the next morning. The animals were anesthetized with 30 mg/kg sodium pentobarbital at day 5 of pregnancy and the entire uterus was removed along with adjacent adipose tissue to include the uterine artery. The uterine horns were separated, rinsed in isotonic saline (0.9%) to remove excess blood. Each horn was then cut into small pieces and fixed for 24 h at room temperature in 10 per cent buffered neutral formalin. Dehydration in ethanol, clearing in xylene and embedding in paraffin were carried out by standard methods. Serial cross-sections 10 μg in thickness were cut and stained with hematoxylin and eosin.

RESULTS

The uterine arteries from young females showed that the internal elastica is very prominent and completely crenated at its circumference (Fig. 1), while in senescent females the internal elastica can be seen to become of greater circumference (i.e. less crenated, see Fig. 2). Also the uterine arteries from senescent females showed extensive distortion of the internal elastica and endothelial proliferation, almost to the point of occlusion of the lumen (Fig. 2).

DISCUSSION

The golden hamsters show a decline in litter size at approximately 14 months of age (Soderwall *et al.*, 1960). the most prominent histological feature in aging of the rodent

uterus is the accumulation of collagen. There is a progressive increase in connective tissue between the two muscle layers, stroma, and around the blood vessels. The amount of collagen increases in the hamster uterus with age (Rahima and Soderwall, 1977). In this study we investigated the structure of uterine arteries from young and senescent females. The uterine arteries from young females showed that the internal elastica is very prominent and completely crenated at its circumference, while in senescent females the internal elastica can be seen to become of greater circumference (i.e. less crenated). Also the uterine arteries from senescent females showed extensive distortion of the internal elastica and endothelial proliferation, almost to the point of occlusion of the lumen. The degenerative uterine arteries, in the case of senescent females, could be interpreted as the medial smooth muscle of mesenchymal cells migrating through gaps in the internal elastica and proliferating within the intima, forming intimal cushions, mucopolysaccharides, and collagen. The material encrusted on the internal elastica could be largely a mixture of calcium and mucopolysaccharides. These degenerative changes in the uterine arteries could lead to the decreased uterine blood flow as reported by Larson and Foote (1972) and Rahima and Soderwall (1977).

Larson and Foote (1972) studied the uterine blood flow in young and aged female rabbits and found that the average uterine blood flow (ml/100 g tissue/min) was higher in young females. Golden hamsters showed the same phenomenon (i.e. the young females had higher endometrial blood flow rate).

The accumulation of collagen in the senescent uterus, as well as the distortion of the uterine blood vessels could lead to the observed reduced uterine blood flow rate. This reduction in senescent females can be interpreted as an important factor which leads to the impairment of the histotrophic substance supply to the developing embryo. This then consequently leads to the observed litter size decline in senescent females.

SUMMARY

The uterine arteries structure was studied in 10 young and eight senescent pregnant female golden hamsters. The uterine arteries from young females showed that the internal elastica is very prominent and completely crenated in its circumference while in senescent females the internal elastica can be seen to become of greater circumference (i.e. less crenated). Also the uterine arteries from senescent females showed extensive distortion of the internal elastica and endothelial proliferation, almost to the point of occlusion of the lumen.

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Vol.
16
1981

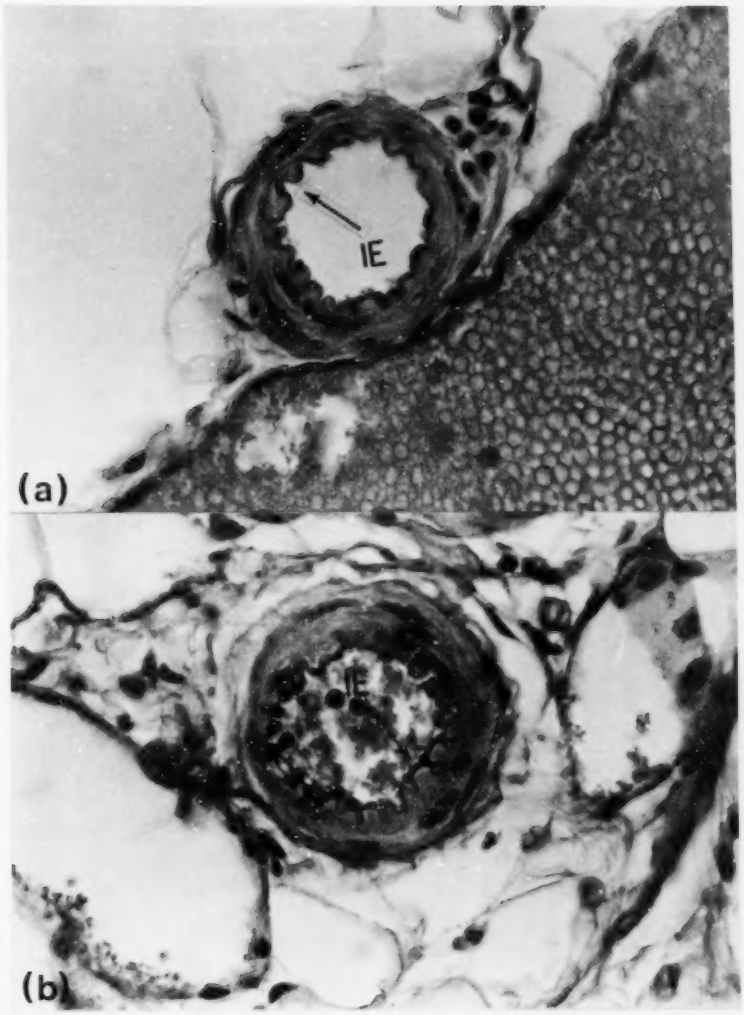

FIG. 1. (a and b) Cross-sections of uterine arteries from pregnant young golden hamster. Note the internal elastica (IE) is very prominent and completely crenated in its circumference.

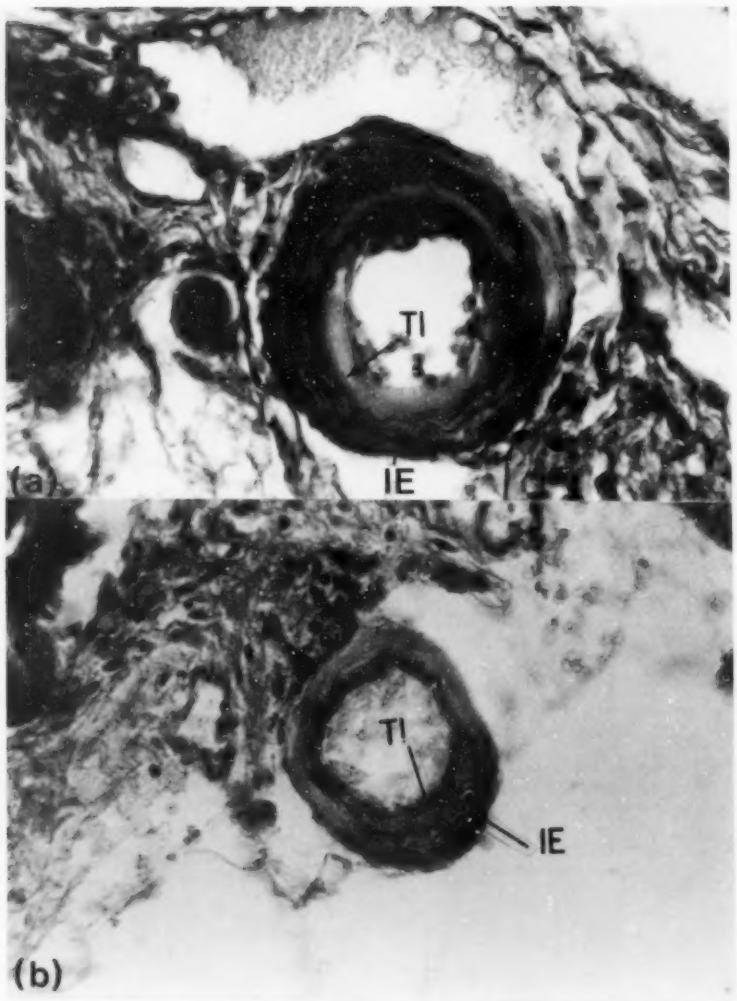

FIG. 2. (a and b) Cross-sections of uterine arteries from pregnant senescent golden hamster. Note that the internal elastica becomes of greater circumference (i.e. less crenated). Also, the tunica imtima (TI) which in young animals was so thin that it cannot be seen, is so prominent in senescent uterine artery (i.e. the endothelial proliferation is so extensive, almost to the point of occlusion of the lumen).

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RELATIONSHIP BETWEEN METABOLIC RATE, LIPOFUSCIN ACCUMULATION AND LYSOSOMAL ENZYME ACTIVITY DURING AGING IN THE ADULT HOUSEFLY, *MUSCA DOMESTICA*

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Vol.
16
1981

INTRODUCTION

ONE OF the most ubiquitous cellular alterations during aging of multicellular organisms is the accumulation of age pigment or lipofuscin in the long-lived postmitotic cells (for references see Brizzee and Ordy, 1981; Miquel *et al.*, 1975*a*; Porta and Hartroft, 1969; Toth, 1968). It is now well established that lipofuscin granules are secondary lysosomes of residual body variety and constitute the partially digested residues of cytoplasmic autophagocytosis (Brandes, 1966; DeDuve and Wattiaux, 1966; Miquel *et al.*, 1975*a*). In addition, there is considerable inferential evidence to suggest that lipid peroxidation and molecular cross-linking reactions initiated by free radical intermediates during aerobic respiration may be involved in the degradation of cell organelles during lipofuscin formation (Miquel *et al.*, 1975*a*; Tappel, 1975, 1980). Due to the involvement of degradative processes lipofuscin formation has been thought to be indicative of the dysfunctional cellular changes during aging (Brandes, 1966; Hothschild, 1968; Strehler, 1964). The question, however, remains whether lipofuscin accumulation is coincidental to aging or it is an integral aspect of aging.

The present study was conducted in order to elucidate, experimentally, the relationship between lipofuscin content, lysosomal enzyme activity and aging using the adult housefly, *Musca domestica* as a model system. The experimental approach employed in this investigation was to alter the lifespans of the flies by varying the levels of metabolic activity. Previous studies in this laboratory have indicated that the average and the maximum lifespan of the housefly and other insects can be extended more than two-fold by restricting physical activity of flies through prevention of flight (Ragland and Sohal, 1972, 1975; Sohal, 1981; Sohal and Buchan, 1981; Sohal and Donato, 1978, 1979). It was postulated that if the changes in the rate of lipofuscin accumulation and levels of lysosomal enzyme activity corresponded to the metabolic rather than chronological age of the flies, then such changes could be considered to be mortality-dependent and maybe specific to the aging process.

MATERIALS AND METHODS

Eggs were obtained from 7 to 10 day old houseflies and placed in moist CSMA (Chemical Specialties Manufacturer's Association) larval medium obtained from Ralston Purina Co., Richmond, IN. After eclosion from the pupae, the adult flies were fed on a mixture of sucrose, dried milk and powdered egg yolk (6:6:1, v/v) and kept throughout life at 25°C with a relative humidity of about 35–40 per cent. Only the male flies were used for experimental studies.

Variation in physical activity

The relative level of physical activity was varied by housing male flies in two different-sized containers. In one group where higher level of physical activity was desired (HA group) flies were put in 1 ft^3 cages where they could fly. In the second group, the level of physical activity was kept low by confining flies individually in a 250 ml glass jar partitioned with a cardboard maze so that the fly could walk but was unable to fly due to restricted space (LA group).

Measurement of physical activity

The levels of physical activity were measured as walking and flying activity simultaneously, but recorded separately, using a radar-Doppler instrument. The principle and the usage of this device in measurement of physical activity in the housefly has been described previously (Buchan and Moreton, 1981; Buchan and Sohal, 1981). In order to measure physical activity in the high activity group a single cage, containing the flies, was placed, one at a time, in a marked position. The movement of the insects was detected by a radar module mounted on the wall of the metal chamber. It should be mentioned that radar-Doppler is a highly sensitive device capable of measuring the movement of single flies even by half their body length.

Measurement of metabolic rate

The oxygen consumption rates of flies were measured in 8-day old male houseflies using S-3A Oxygen Analyzer (Applied Electrochemistry Inc., Sunnyvale, California). The high activity flies were placed in a 2.6 l. clear plastic respiration chamber in which the flies were free to fly. In the case of the low activity group, each fly was confined within a separate vial of about 25 ml capacity. The vials, containing the flies, were then transferred to the respiration chamber. Concentration of oxygen in the respiration chamber was recorded over a four hour period.

Electron microscopy

Lipofuscin volume was determined morphometrically in the epithelial cells of midgut and oenocytes from the fat body because previous studies in this laboratory had indicated that these cell types accumulate considerable amounts of lipofuscin with age (Sohal, 1973; Sohal and McCarthy, 1973). Midgut and fat body from houseflies of different ages were dissected out and fixed in 2.5% glutaraldehyde buffered with phosphate, followed by post-fixation in 1% phosphate-buffered osmium tetroxide, dehydrated in alcohol and embedded in Maraglas. Tissue sections were stained with uranyl acetate and lead citrate and were randomly photographed at a magnification of about 10,000 by a Hitachi HU11B electron microscope. The negatives were further enlarged 2.5 times. The percentage area of the cytoplasm occupied by lipofuscin was determined by differential point counting method (Weibel, 1969) from 30 to 40 micrographs in each group. Student's t test was used in determining the significance of the differences between various groups. Differences at 0.05 level were considered to be statistically significant.

Lysosomal enzyme activity

In each group, 16 flies were homogenized in 8 ml of 1% Triton X. The homogenates were centrifuged at 20,000 g for 30 min. The supernatant was sonicated by three separate bursts each of 30 s duration and then used for enzyme assays. Activities of β-glucoronidase, β-glycerophosphatase and ribonuclease were measured according to the procedure outlined by Barret (1972) using phenolphthalein β-glucosiduronate, β-glycerophosphate and yeast RNA respectively, as substrates (Sigma Chemicals).

RESULTS

Life spans, physical activity and metabolic rate

The lifespans of the housefly populations kept in 1 ft^3 cages where flying was possible (high activity) and those confined individually within small bottles which were unable to fly (low activity) are presented in Table 1. The average and the maximum lifespans of low activity flies were more than two-fold longer than in the high activity flies.

The walking and flying activity of the high and low activity groups was determined in 8-day old male flies. The walking activity of the flies in the two groups was quite similar. No flying activity was detected in the low activity flies whereas the high activity flies exhibited a considerable level of flying (Table 1).

A comparison of oxygen consumption rates in 8-day old male flies indicated that the high activity flies consumed about 50% more oxygen than the low activity flies (Table 1).

TABLE 1. LIFESPANS, PHYSICAL ACTIVITY* AND OXYGEN CONSUMPTION* RATES IN HIGH ACTIVITY (HA) AND LOW ACTIVITY (LA) ADULT MALE HOUSEFLIES

Group	Lifespan		Walking activity (counts/h/fly)	Flying activity (counts/h/fly)	Oxygen consumption (μl/h/mg wet wt)
	Average	Maximum			
HA	16.2 ± 2	33	52 ± 3	289 ± 4	11.2 ± 1.2
LA	38.5 ± 2.5	78	50 ± 2	0	7.6 ± 1.0

Values are ± S.E.M.
*Measured in 8-day old male flies.

Lipofuscin content

In order to avoid sampling errors due to selective mortality, the lipofuscin volume and lysosomal enzyme activity were determined in 1–14-day old flies. The 14-day old high activity flies were considered to be roughly at the same metabolic age as the 30-day old low activity flies because these ages represented a period when around 20 per cent mortality had occurred in both populations.

Epithelial cells of the midgut and the oenocytic cells in the fat body of the houseflies of different ages were examined for lipofuscin content. Age associated changes in the fine structure of these cell types have been reported previously from this laboratory (Sohal, 1973; Sohal *et al.*, 1977).

Midgut. The epithelial cells form a single cell thick layer around the lumen of the midgut and exhibit a fairly uniform structural organization. Their cytoplasm is rich in rough endoplasmic reticulum, free polysomes and contains numerous mitochondria as well as well developed Golgi complexes. In 1-day old flies only a relatively small volume of the cytoplasm is occupied by lipofuscin and other lysosomal structures identified on the basis of their positive localization of acid phosphatase activity as reported previously (Sohal *et al.*, 1977). However, there was a tremendous increase with age in the lipofuscin content of the cells (Figs. 1, 2; Table 2). Lipofuscin granules were easily distinguishable, morphologically, from other organelles. They are bounded externally by a single membrane and contain a highly electron dense amorphous-looking component and a pleomorphic membranous part. A quantitative comparison of lipofuscin in the epithelial cells of the midgut of 1-, 7-, and 14-day old high and 1-, 7-, 14-, and 30-day old low activity flies indicated that the lipofuscin content increased with age in both groups ($p < 0.001$), however, the high activity flies contained significantly more (~50 per cent) lipofuscin than low activity flies at both day 7 as well as day 14 of age indicating that the rate of lipofuscin accumulation was faster in the high than in the low activity flies. There were no statistically significant differences in the amount of lipofuscin in the epithelial cells of 30-day old low activity and 14-day old high activity flies.

Fat body. The fat body in the housefly is composed of two types of cells, viz, fat cells and oenocytes (Sohal, 1973). The latter were examined in this study because they exhibit substantial increase in lipofuscin content with age as mentioned above. Oenocytes are morphologically characterized by the relative abundance of smooth endoplasmic reticulum and free polysomes. Rough endoplasmic reticulum is quite sparse. A comparison of the lipofuscin content of the oenocytes from flies of different ages belonging to high and low activity groups indicated that lipofuscin content increased with age (Table 2) in both groups of flies ($p > 0.001$) but the rate of accumulation was significantly faster in high activity flies as compared to the low activity flies ($p > 0.05$). The relative levels of lipofuscin were, however, similar in 14-day high activity and 30-day old low activity flies.

Vol. 16 1981

TABLE 2. LIPOFUSCIN VOLUME IN EPITHELIAL CELLS OF MIDGUT AND OENOCYTES OF THE FAT BODY IN ADULT HOUSEFLIES OF DIFFERENT AGES KEPT UNDER HIGH (HA) AND LOW ACTIVITY (LA) REGIMES

Age of flies (days)	Group	Lipofuscin (% cellular volume) Midgut	Fat body
1		0.9 ± 0.15	0.7 ± 0.2
7	HA	8.2 ± 1.1	7.8 ± 1.2
	LA	5.2 ± 0.8	4.2 ± 0.5
14	HA	18.1 ± 2.0	11.8 ± 1.1
	LA	11.9 ± 0.9	7.3 ± 1.1
30	LA	16.3 ± 1.2	12.5 ± 1.5

Values are ± S.E.M.

Lysosomal enzyme activity

β-glucoronidase. The profile of the activity of this enzyme was somewhat different in the two groups of flies (Table 3). In the high activity flies enzyme activity showed a slight increase between 1–5 days of age and then gradually declined in the 9- and 14-day old flies. In the low activity flies there was a gradual decrease in the enzyme activity with age beginning after day 1. At all ages the enzyme levels in low activity group were below those in the high activity flies.

TABLE 3. LYSOSOMAL ENZYME ACTIVITY IN THE WHOLE BODY HOMOGENATES OF ADULT HOUSEFLIES OF HIGH (HA) AND LOW ACTIVITY (LA) GROUPS AT DIFFERENT AGES

Enzyme	Group	Age (days) 1	5	9	14	30
β-glucoronidase (μmoles/g wet wt/h)	HA	80 ± 3	91 ± 3	83 ± 2	74 ± 2	
	LA		74 ± 4	70 ± 3	62 ± 2	54 ± 4
β-glycerophosphatase (μmoles/g wet wt/h)	HA	33.6 ± 3.5	30.4 ± 2.6	28.7 ± 4.2	32.6 ± 3.3	
	LA		26.5 ± 3.1	28.5 ± 1.8	29.5 ± 4.2	34.6 ± 2.6
ribonuclease (mg RNA-ase × 10^{-3}/mg wet wt)	HA	32.7 ± 3.5	31.1 ± 2.0	24.2 ± 3.6	16.5 ± 2.7	
	LA		27.4 ± 2.8	20.2 ± 2.1	15.0 ± 1.4	14.1 ± 1.1

Values are ± S.E.M.

β-glycerophosphatase. The activity of this enzyme did not significantly alter with age of the flies, nor were there any significant differences between the high and the low activity flies (Table 3).

Ribonuclease. There was a gradual decrease in the activity of this enzyme with age in both groups of flies (Table 3). There were, however, no significant differences between high and low activity flies of the same age.

DISCUSSION

The results of the present study indicate that the rate of lipofuscin accumulation in two different cell types is stimulated by an increase in the metabolic rate and corresponds

Vol.
16
1981

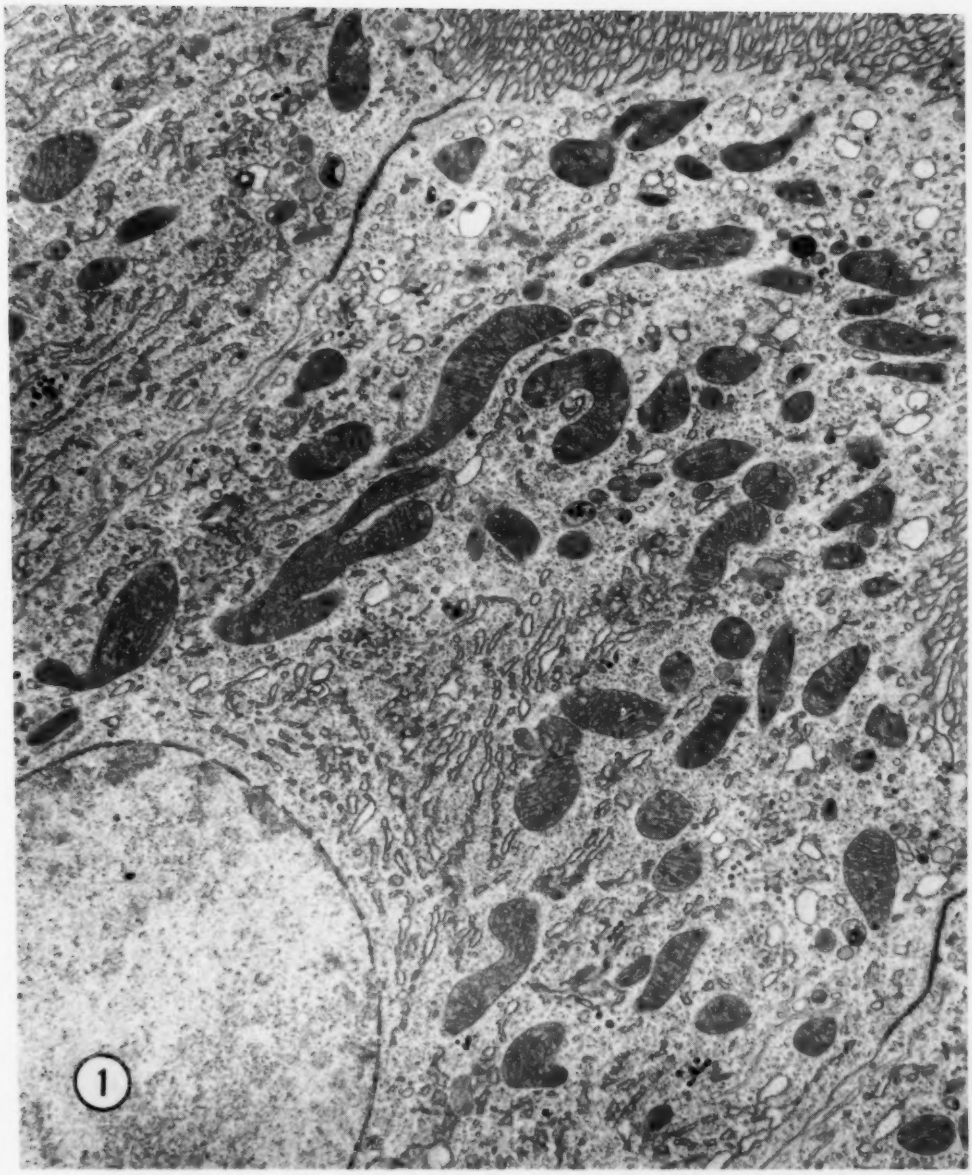

FIG. 1. The epithelial cells of the midgut of a one-day old adult male housefly. × 14,000.

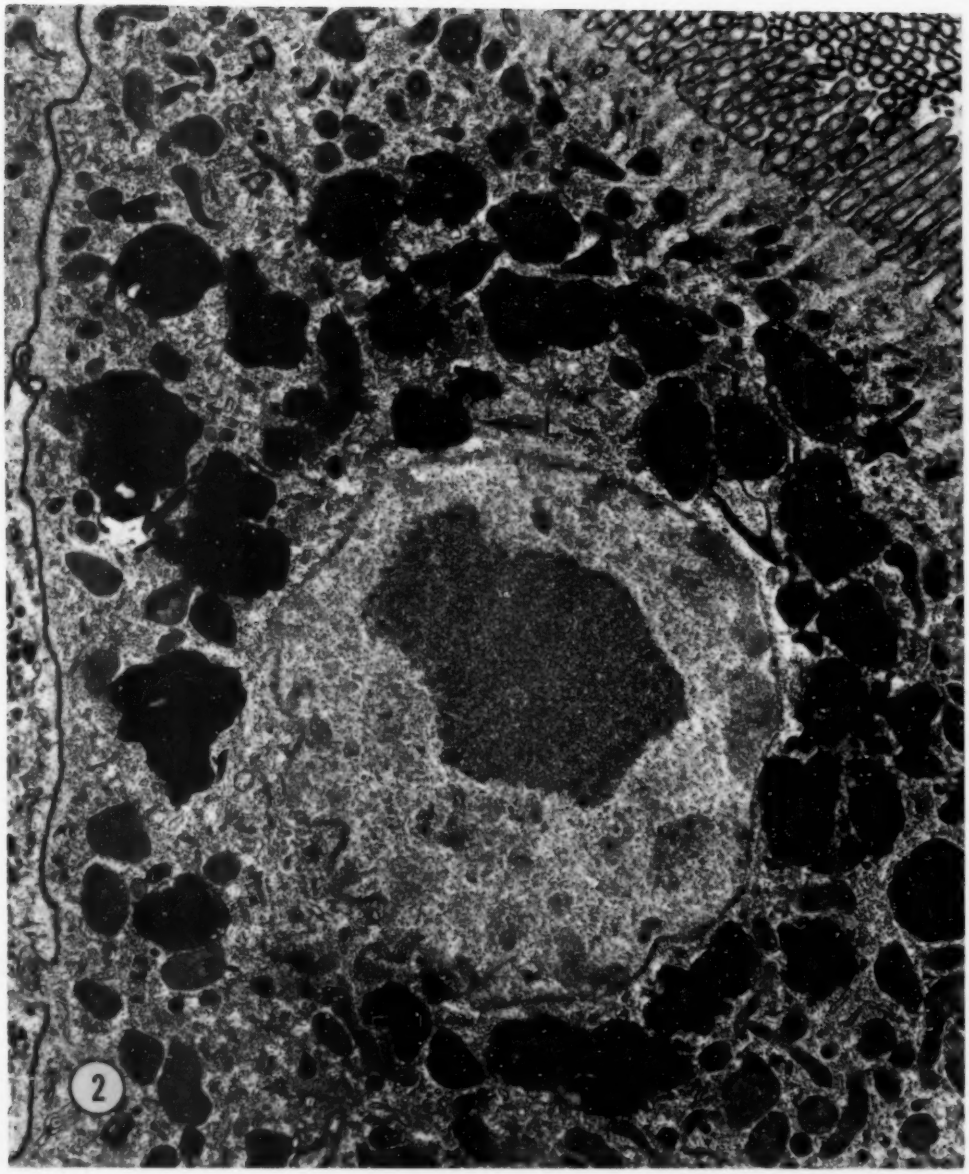

FIG. 2. Section through an epithelial cell of the midgut from a 14-day old male housefly, belonging to high activity group, showing the accumulation of lipofuscin (L) granules. These structures also contain a high concentration of minerals (Sohal *et al.*, 1977). × 25,000.

to the consequent shortened lifespan of the houseflies. Activities of the three lysosomal enzymes, however, exhibited distinct differences (e.g., β-glucoronidase is affected by both age and physical activity), whereas ribonuclease is influenced by age alone and β-glycerophosphatase is not affected by either age or level of physical activity.

The mechanism by which metabolic rate affects lifespan and lipofuscin formation is as yet unclear; however, there is considerable inferential evidence to suggest that free radicals generated as a result of univalent reduction of oxygen may be involved in aging as well as lipofuscin formation (for reviews see Donato, 1981; Fridovich, 1978; Halliwell, 1981; Leibovitz and Siegel, 1980; Miquel *et al.*, 1975*a*). Statistical analyses of mortality rates of high and low activity houseflies, involving Gompertz function, have suggested that alterations in metabolic rate may affect lifespan by modulating the rate of aging process (Sohal, 1981). It has been postulated that free radicals cause membrane damage by lipid peroxidation and the breakdown products of lipid peroxides, chiefly malonaldehyde can cross-link amine containing molecules such as proteins, nucleic acids and some phospholipids to produce Schiff base fluorescent compounds present in the lipofuscin granules (Tappel, 1975). Since lipofuscin granules also include undigested endproducts of autophagocytosis, the results of the present study tend to imply that an increase in metabolic rate may be considered to stimulate the process of cytoplasmic autophagocytosis. An age-associated increase in the relative percentage area of the cytoplasm occupied by lipofuscin, as observed here, also suggests a proportionate degeneration and loss of cytoplasmic organelles. It is possible that increased autophagocytosis in response to higher metabolic activity is due to enhanced organelle damage because of greater production of free radicals. Increase in autophagocytosis and lipofuscin formation has been noted previously in response to pathological conditions promoting lipid peroxidation (Wolman, 1981). Similarly, an increase in the atmospheric oxygen concentration (Miquel *et al.*, 1976) and ambient temperature (Miquel *et al.*, 1975*b*) have also been found to increase the rate of lipofuscin formation in drosophila. It, therefore, seems that a relationship exists between rate of oxygen consumption, lipofuscin accumulation and the aging process, albeit, the underlying mechanisms need to be elucidated.

Despite the fact that lipofuscin is a component of the lysosomal system, the increase in its volume with age is not accompanied by a corresponding increase in the activity of lysosomal enzymes in the houseflies. Furthermore, the activities of various lysosomal enzymes may not be related to the volume of lipofuscin as indicated by the finding that the β-glycerophosphatase and ribonuclease levels remain at relatively similar levels in high and low activity flies whereas the amount of lipofuscin in the two groups appears to be different, at least, in the midgut and fat body. A similar discrepancy was also found between the lipofuscin content of the giant nerve cells of the brain and lysosomal enzyme activity in the head of the housefly (Sohal and Donato, 1979).

Another notable feature of the present study is that different lysosomal enzymes apparently vary in their age-associated expression and inducibility in response to external influences. Whereas the activities of β-glycerophosphatase and ribonuclease remained either unchanged or declined with age and did not respond to alterations in physical activity, β-glucoronidase activity, on the other hand, initially increased with age in high but not in low activity flies. At all ages the high activity flies exhibited a higher level of β-glucoronidase activity. The reasons for the differential inducibility of this enzyme by higher metabolic rate are unclear and cannot be explained on the basis of the present results. It is possible that increase in β-glucoronidase activity may be due to greater availability of the substrate.

Vol. 16 1981

In conclusion, it can be stated that metabolic rate affects the rate of age-associated accumulation of lipofuscin whereas the response of different lysosomal enzymes to variation in the metabolic rate is quite selective. Lipofuscin appears to be a cellular marker of the metabolic age of the flies. The view that aging involves higher lysosomal activity (Hothschild, 1971) cannot be supported as a general occurrence.

SUMMARY

The objective of this study was to elucidate experimentally the significance of lipofuscin accumulation and lysosomal enzyme activity during the aging process. Average and maximum lifespans of male houseflies were extended more than two-fold by restricting flight activity. Levels of physical activity, measured by radar-Doppler system, and oxygen consumption rates were lower in the long-lived low activity flies as compared to the short-lived high activity flies. Rate of lipofuscin accumulation determined by morphometric techniques, in epithelial cells of the midgut and oenocytes of the fat body was greater in high activity than in low activity flies. The activity of β-glycerophosphatase was unaffected by age and physical activity whereas ribonuclease levels decreased with age but remained unchanged by physical activity. Levels of β-glucoronidase were lower in low activity flies than in high activity flies. The results of this study suggest that metabolic rate accelerates the rate of lipofuscin accumulation while shortening the lifespan of houseflies and has a variable effect on the activity of different lysosomal enzymes.

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Vol.
16
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DIETARY RESTRICTION REDUCES FLUORESCENT AGE PIGMENT ACCUMULATION IN MICE

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Vol. 16 1981

INTRODUCTION

DIETARY RESTRICTION has been reported to significantly extend the lifespan of rats and of mice (McCay *et al.*, 1939; Ross, 1959; Barrows and Roeder, 1977). One means of imposing dietary restriction is to decrease the level of dietary protein (Barrows and Kokkonen, 1977). Leto *et al.* (1976) and Goodrick (1978) have both reported that the lifespan of mice on a 4 per cent protein diet is significantly longer than that of control mice on a 26 per cent protein diet.

Other studies have shown that fluorescent age pigment, or lipofuscin, increases significantly in neurons and other tissues as animals age (Brizzee *et al.*, 1975; Gordon, 1974). Lipofuscin accumulation with age has been demonstrated both by histological means (Reitchel, 1968) and by spectrofluorometric measurements of lipid peroxidation fluorescent products (Tappel *et al.*, 1973; Miguel *et al.*, 1978). A correlation between age and the accumulation of lipofuscin has been demonstrated in heart muscle (Strehler *et al.*, 1959; Munnell and Geity, 1968), in brain, adrenal cortex, kidney, testis (Reichel, 1968) and in liver (Bachmann, 1953; Goldfisher and Bernstein, 1969). Lipofuscin is present in tissues of newborn animals, and accumulates linearly with age.

If animals on dietary restriction age more slowly than control animals, we might expect that lipofuscin would accumulate more slowly in these animals.

No studies had been carried out thus far on the relationship between dietary restriction and lipofuscin accumulation. Therefore, the question that we set out to investigate was as follows: Do potentially longer-lived mice on a restricted diet have a slower rate of lipofuscin accumulation than fully-fed control mice? We were also interested in determining whether differences in the level of fluorescent age pigment could be observed as a function of diet in young mice, or whether differences would be apparent only in old animals.

MATERIALS AND METHODS

Animals and diets

Male Swiss albino mice were obtained from Canadian Breeding Farms, St. Constant, Québec at 3 weeks of age. They were maintained on Purina chow for 1 week, until they had attained an average body weight of 15 g. At 1 month of age, groups of control mice were placed on Teklad diet TD78071 containing 26 per cent protein, while the other groups intended for restriction were placed on Teklad diet TD78072, containing 4 per cent protein. These diets are produced by Teklad Co., Madison, Wisconsin. The protein source in these test diets is casein and the diets are isocaloric. The diets were available to the mice *ad libitum*. The construction of the diets is described by Leto *et al.* (1976).

Determination of lipid peroxidation fluorescent products

Lipid peroxidation products were extracted and their fluorescence level was quantitated using the method of Tappel *et al.* (1973). Determinations were carried out on brain and heart for mice of 3, 5, 7 and 12 months of age. Tissues from three or four mice were used for determinations at each age and each diet. The brain

and heart ventricles were excised, the tissues were macerated and washed repeatedly in 0.9% saline solution to remove excess blood. The 0.2 g tissue samples were then placed in 4 ml of chloroform : methanol prepared 2 : 1 (v/v).

An extraction time of 1 min was used for brain tissue but 1.5 min was used as an extraction time for the fibrous heart tissue. Samples were homogenized at 1300 rpm for 1 min in a Potter–Elvjhen type homogenizer and then 4 ml of de-ionized water was added to the homogenate. The chloroform layer was separated by centrifugation at 700 g for 10 min in a clinical centrifuge. The water layer was removed by aspiration and pipetting, while the chloroform layer was filtered through Whatman No. 42 filter paper prior to analysis.

Fluorescence measurements were made using an Aminco-Bowman spectrophotofluorometer standardized with quinine sulfate (1 mg/ml of 0.1 M H_2SO_4). Measurements were taken with the voltage set at 700 V and wavelength set at excitation 358 nm and emission 438 nm. The slit opening was 5. Measurements were obtained in fluorescent units per 0.2 g tissue sample.

Statistical analysis

Data was analyzed by means of the standard *t*-test.

Determination of whole body and organ weight

Whole body weight was determined on separate groups of 160 animals on the control and on the restricted diet respectively. The fresh organ weight of brain and heart is the average of 20 animals per group.

RESULTS

Table 1 shows that the level of lipid peroxidation fluorescent products is significantly lower in the brain tissue of mice on the restricted diet as compared to those on the control diet for all age groups measured. Even in the youngest group, at 3 months of age, the difference in level of fluorescent product is significantly lower in the brain of the animals on dietary restriction. By 12 months of age, the mice on the restricted diet have 52 per cent less lipid peroxidation fluorescent products per 0.2 g tissue sample than do mice on the control diet. These results for brain tissue are shown graphically in Fig. 1, which emphasizes both the steady increase in lipid peroxidation fluorescent products in brain tissue with age, and the consistently higher level of these fluorescent products in the brains of mice on the control diet.

TABLE 1. DATA FOR BRAIN. COMPARISON OF MEAN FLUORESCENCE UNITS REPRESENTING LIPID PEROXIDATION FLUORESCENT PRODUCTS FOR 0.2 g OF BRAIN TISSUE FROM MICE MAINTAINED ON RESTRICTED DIET AND ON CONTROL DIET AT 3, 5, 7 AND 12 MONTHS OF AGE. THE ASTERISK (*) INDICATES A SIGNIFICANT ($p < 0.05$) INCREASE IN THE FLUORESCENCE LEVEL AS COMPARED TO THE PREVIOUS AGE GROUP

Age in months	Restricted diet (fluorescence units ± S.E.)	Control diet (fluorescence units ± S.E.)	Level of significance	% Difference between restricted and control
3	30.00 ± 7.85	38.63 ± 4.11	$p < 0.05$	28.7
5	38.29 ± 7.09*	53.25 ± 4.60*	$p < 0.01$	39.1
7	60.00 ± 7.56*	70.75 ± 6.92*	$p < 0.02$	17.9
12	53.77 ± 3.62	81.63 ± 17.11	$p < 0.01$	51.8

Table 2 shows that the level of fluorescent product is always lower in samples of heart tissue for mice on the restricted diet as compared to those on the control diet. However, the difference between the two groups is not statistically significant until the animals are 12 months of age. At 12 months, the level of lipid peroxidation fluorescent products is 25 per cent lower in the heart tissue of mice on the restricted diet. Thus, for the heart tissues there is no influence of diet on this parameter until the animals are 12 months of age.

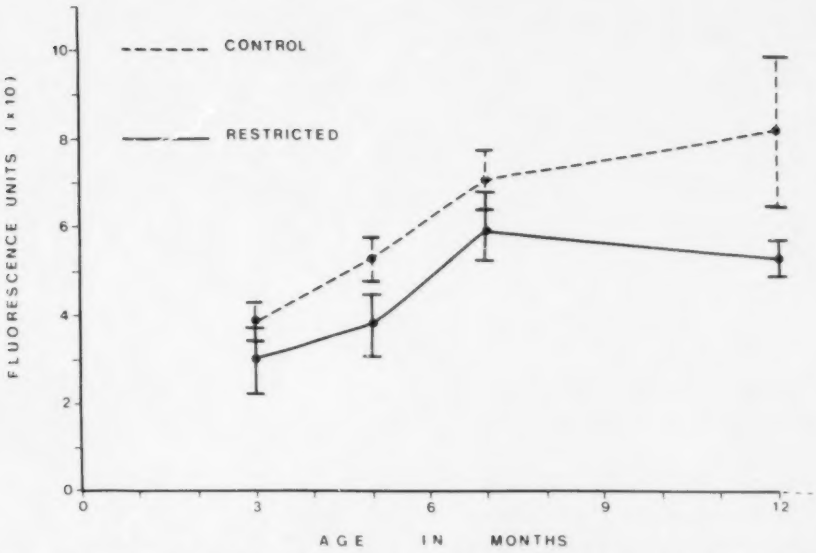

FIG. 1. In the brain, the level of lipid peroxidation fluorescent products, measured in fluorescence units per 0.2 g of tissue increases with age. The level is consistently lower in the brains of mice on the restricted diet of 4 per cent protein as compared to the brains of mice on the control diet.

Vol. 16 1981

TABLE 2. DATA FOR HEART. COMPARISON OF MEAN FLUORESCENCE UNITS REPRESENTING LIPID PEROXIDATION FLUORESCENT PRODUCTS FOR 0.2 g OF HEART TISSUE FROM MICE MAINTAINED ON RESTRICTED DIET AND ON CONTROL DIET AT 3, 5, 7 AND 12 MONTHS OF AGE

Age in months	Restricted diet (fluorescence units ± S.E.)	Control diet (fluorescence units ± S.E.)	Level of significance	% difference between restricted and control
3	38.33 ± 1.72	39.03 ± 0	NSD	2.5
5	43.50 ± 9.13	48.05 ± 0	NSD	10.5
7	50.25 ± 22.82	52.50 ± 9.12	NSD	3.8
12	47.45 ± 4.60	59.45 ± 31.94	$p < 0.05$	25.3

The increase in lipid peroxidation fluorescent products that we observe as a function of age in both brain and heart tissue of mice in both dietary groups is not linear with age in all cases. It is well known that there may be considerable variability in lipid peroxidation fluorescent product measurements (Tappel *et al.*, 1973).

To emphasize that there is a considerable difference in size between animals on the control and on the restricted diet, the data for whole body weight and for organ weight is presented in Table 3. The whole body weight of animals on the restricted diet is only half that of animals on the control diet. Brain weight is maintained in restricted animals at around 90 per cent of control, while heart weight of animals on the restricted diet is around 60 per cent of the control weight.

TABLE 3. THE WEIGHT OF THE WHOLE BODY, OF THE BRAIN AND OF THE HEART FOR MICE ON THE RESTRICTED DIET ARE COMPARED TO MICE ON THE CONTROL DIET AT 3, 5, 7 AND 12 MONTHS OF AGE

Age in months	Whole body weight, g (N = 160)		Weight of brain, g (N = 20)		Weight of heart, g (N = 20)	
	Restricted	Control	Restricted	Control	Restricted	Control
3	18.66	38.15	0.398	0.431	0.110	0.173
5	21.14	45.97	0.412	0.454	0.122	0.206
7	22.64	51.12	0.421	0.469	0.130	0.228
12	25.04	59.37	0.436	0.493	0.143	0.263

DISCUSSION

Our results show that potentially longer-lived mice on dietary restriction have lower levels of lipid peroxidation fluorescent products than do control mice on an unrestricted diet.

In the case of the brain, the difference is apparent as early as 3 months of age. In heart tissue, a significant difference is seen only at 12 months of age.

In interpreting our results, we must ask whether lipofuscin accumulation is influenced by the rate of aging, whether it is influenced by diet, and how much these factors are interrelated.

The relationship between lifespan and lipofuscin levels has been examined by Biscardi and Webster (1977). Using *Drosophila,* they studied the question of whether a single gene mutation which reduces lifespan can also influence lipofuscin level. They reported that the rate of fluorescent pigment accumulation in wild type *Drosophila melangaster* was the same as the rate in two short lived mutant strains. Thus, a genetically determined reduction in lifespan did not influence the rate of lipofuscin accumulation in this case.

Although drugs, such as centrophenoxine (Nandy and Bourne, 1966; Hochschild, 1973) and antioxidants such as vitamin E and butylated hydroxytoluene (Tappel *et al.,* 1973) have both been reported to retard or reduce lipofuscin build up, there have been fewer studies on the influence of diet on lipofuscin accumulation.

Concerning diet, Tauchi *et al.* (1980) have shown that race and environment may influence lipofuscin accumulation in hepatic cell in humans. The amount of hepatic cell lipofuscin was larger in persons of Japanese origin living in Hawaii than in those living in Japan. These authors suggest that the richness of the diet may be a contributing factor.

Harman (1978) reports that increasing the dietary content of either unsaturated fat or easily oxidized amino acids increases the level of free-radical reactions and consequently decreases lifespan. Since lipofuscin build up is thought to result from free-radical reactions, a difference in the level of free-radical reactions, produced by the two diets could explain our data. According to this interpretation, the increased level of protein provided to the control animals would contribute to an increased level of free-radical reactions, which in turn would result in an increased build up of lipofuscin.

Lamb (1977) suggests the possibility that dietary restriction may prolong life primarily by preventing deleterious effects of obesity. Our control animals are clearly much larger than our experimental animals, as indicated in Table 3. Our dietary regime clearly restricted the growth of the experimental animals from the earliest age that they were placed on the diet. Although the control animals are larger, and subjectively appear somewhat obese, we do not yet have a measure of relative obesity of the two groups of animals. It

would be of interest to pursue the line of thought that lipofuscin level is correlated with obesity.

SUMMARY

The influence of dietary restriction of lipid peroxidation fluorescent products, or lipofuscin, was examined in brain and heart in male Swiss albino mice at 3, 5, 7 and 12 months of age. The level of fluorescent products was significantly lower in the brain of mice on the restricted diet as compared to control mice in all age groups. By 12 months of age, the level of lipid peroxidation fluorescent products was significantly lower in the heart tissue of mice on the restricted diet as compared to control mice. Dietary restriction may retard lipofuscin accumulation by reducing the level of free radical-reactions.

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Vol.
16
1981

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TIME COURSE OF HORMONAL RESPONSE TO SEXUAL BEHAVIOR IN AGING MALE RATS

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INTRODUCTION

MATURE MALE rats respond to sexual behavior with increased concentrations of plasma testosterone, LH and prolactin (Kamel *et al.*, 1975, 1977; Kamel and Frankel, 1978). We recently reported that 27-month old male rats neither mate nor demonstrate a hormonal response to a receptive female (Frankel, 1981). However, these animals were sexually naive at the time the experiment began. Further, three different blood samples (basal, 15 and 50 min after entrance of a receptive female) were collected by cardiac puncture from anesthetized rats on three different days. We therefore repeated the study with sexually experienced aged rats, and compared their behavior and hormonal response with younger animals. Blood was collected from indwelling jugular cannulas in unanesthetized, unhandled animals, five successive times after entrance of a receptive female. Plasma concentrations of testosterone, LH and prolactin were measured. Behavior was monitored by keying into a computer which was programmed to time, store and analyze parameters to sexual activity.

METHODS AND PROCEDURE

Animals

Male Sprague-Dawley rats were reared in our laboratory. At 30–35 days of age, they were caged in pairs in a temperature-controlled room (21 ± 1 C), maintained on a reversed light:dark cycle (lights off, 1000–2200 h). Purina rat chow and water were available *ad libitum*. Two-year old animals in the study were breeders in our colony, were retired at 1 year of age, and entered the experiment January 1980. They were given sexual experience once a month (starting at 19 months of age). During April and May, their sexual behavior was tested twice. In June their jugular vein was cannulated, and three days later, blood was collected while their sexual behavior was monitored. Younger animals were treated similarly, except that they were given sexual experience monthly from 65 days of age.

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Experimental protocol

Three days prior to the experiment, rats were anesthetized with ether, and a cannula was inserted in the right jugular vein with the tip in the right atrium, as previously described (Kamel and Frankel, 1978). Immediately after cannulation, rats were placed in a rectangular plexiglass 27 × 53-cm cage, equipped with a cannular flow-through swivel. The rats wore harnesses which were attached to the swivels by 36-cm springs through which the cannulas were threaded. The cannulas were thus protected while allowing the rats to move freely and mate normally. Three days later each rat was bled through its cannula 5 times during a 75-minute period between 1200–1630 h. They were bled 15 min before, and 5,15,30 and 60 min after a receptive female was placed in the male's cage. Blood samples (0.75 ml) were collected into heparinized syringes. After each blood collection, rats were injected through the cannulas with 0.75 ml heparinized saline (10 U/ml).

Behavioral procedures

Behavioral procedures, including those involved in blood collection sessions, were conducted in the animal room under dim red light. Females were brought into sexual receptivity by chronic sc implantation of 5-mm Silastic capsules containing estradiol benzoate and sc injection of 1.0 mg progesterone in sesame oil 4–6 h before use.

A session of sexual experience consisted of a 5-min adaptation period, after which the receptive female was placed in the male rat's cage for 45 min. Sexual behavior tests were carried out in semicircular plexiglass mating arenas (41 × 77 cm). Following a 5-min adaptation period, the female was dropped into the cage and left until one of 4 conditions terminated the test of aged rats: (1) 45 min without an intromission; (2) 60 min without an ejaculation; (3) first intromission after first ejaculation; (4) maximum time limit 67 min. Termination of behavioral tests in younger animals was similar except that conditions were (1) 15 min; (2) 30 min; and (4) 37 min. Behavior was scored with the assistance of a computerized keyboard containing 3 keys operated by the left hand (mount, intromission and ejaculation); and 4 keys by the right hand (animal cages 1, 2, 3, 4). An additional key operated by the right thumb was used to correct an error in scoring during the test. Behavioral events were registered by a North Star Horizon microcomputer located outside the vivarium and appeared visually on the screen monitor above the animal arenas. At the close of an experiment, a summary for each animal was automatically printed out on a Model 43 Teletypewriter. The summary included latencies and frequencies for each series (if more than one ejaculatory series was scored). The design of this installation including a description of the software program has been reported elsewhere (Frankel, 1981).

Hormone assays

Plasma was separated by centrifugation of the blood samples and stored at −20 C until analysis. Hormones were measured by radioimmunoassay: LH by the method of Niswender *et al.*, 1968 and prolactin by use of the NIAMDD rat assay kit. Both methods have been characterized in a previous report from this laboratory (Kamel *et al.*, 1977). In this experiment, sensitivity of the LH radioimmunoassay was 1.03 ng, so that in a 100μl sample the sensitivity of the assay was 10.3 ng/ml. In aged male rats, in which plasma LH was particularly low (probably due to stress of surgery and restrictive harness, see later discussion), those concentrations which were at or slightly beneath the sensitivity of the assay were registered as 10.3 ng/ml. Testosterone was measured by a radioimmunoassay developed in this laboratory (Frankel *et al.*, 1975).

Statistics

Hormone concentrations were evaluated with analysis of variance and further comparisons with the Dunnett test (Keppel, 1973). Analysis of behavioral data was performed using the Wilcoxon two-sample test (Keppel, 1973). Results on 14-month old rats were not treated statistically, due to the small sample size.

RESULTS

Younger rats (9-month old) mated normally, both in scoring tests prior to blood collection and during blood collection (Table 1), although latencies increased significantly ($p < 0.05$) during blood collection. Older rats seldom mated, either prior to or during blood collection. Although all three 14-month old rats displayed sexual behavior during blood collection, one of the three did not mate to ejaculation. Only one of the 24-month old rats (#7) mated during blood collection (Table 2). However, in a test 1 month before, two aged rats (#s 7 and 15) mated and another (#3) displayed some sexual behavior.

Hormones rose significantly during mating in the 9-month old group (Table 3).

TABLE 1. SEXUAL BEHAVIOR BEFORE AND DURING BLOOD COLLECTION BY INDWELLING CANNULA IN 9-MONTH OLD MALE RATS

	ML	IL	EL	PEI
Before blood collection	0.41 ± 0.10	1.52 ± 0.32	7.24 ± 0.88	4.45 ± 0.20
During blood collection	2.28 ± 0.57	4.49 ± 1.46	14.74 ± 3.94	7.01 ± 0.77

The same animals ($n = 7$) were scored twice during April and May, and once while blood was being collected in June. All latencies (in min, mean ± SEM) were significantly higher during blood collection ($p < 0.05$).

Testosterone rose significantly at 5 min and reached its maximum at 60 min; LH at 5 and 15 min and prolactin at 5 and 15 min respectively (Table 3). Similar results were observed in 14-month old rats, although these data were not analyzed statistically. As a group, there was no significant rise in any of the three hormones in the 24-month old. However, in an analysis of individual aged rats in which an increase of hormone concentration was arbitrarily considered to be of importance if it increased 50% above basal levels, two rats (#s 7 and 15) displayed a significant increase in all three hormones (Table 2). One of them, #7, mated to ejaculation during the blood regimen; the other, #15, did not at that time, but did in the last behavioral test prior to blood collection, albeit slowly (Table 2). In 9-month old rats, by the same test of significance, testosterone rose in all 7, prolactin, 6 of 7, and LH, 6 of 7.

Basal levels of testosterone were significantly lower in aged rats, as compared to 9-month old rats ($p < 0.001$). Basal levels of the other two hormones were similar in both age groups (Table 3).

The rank of basal hormone concentrations in aged male rats was examined in order to determine whether such an analysis would offer a predictive evaluation of either sexual behavior and/or hormonal response to sexual behavior. There were no obvious relationships, although the two mating animals in question, #s 7 and 15, ranked high in testosterone and LH, and low in prolactin (Table 2).

DISCUSSION

The results in this study are in close agreement with a previous study (Frankel, 1981) in which blood was collected singly on three different days by cardiac puncture from ether-anesthetized, sexually inexperienced aged male rats. In both reports, hormones rose significantly in younger animals during mating, while there was no hormonal response in older, non-mating animals. However, in this study the aged group included one mater, whose hormones responded to sexual activity. In addition, in this study the ability to examine an individual's plasma hormone concentrations in 5 sequential samples during a 75-min period was a marked advantage over single samples over a 3-day period. This was particularly true in the examination of senescent males whose hormonal response, if any, to a receptive female might be aberrant. The single remaining mater at the time blood was collected in this study was not in itself significant. The animals in this study were approximately 3 months younger than in the previous report. At this age, sexual behavior is failing precipitously.

The practice of using indwelling cannulas attached to restrictive harnesses in order to collect blood samples sequentially from unanesthetized and unhandled animals presents an interesting dilemma. Its obvious advantages must be weighed against an adverse effect

Vol. 16 1981

TABLE 2. ANALYSIS OF INDIVIDUAL AGED RATS: BEHAVIOR BEFORE AND DURING BLOOD COLLECTION AND ITS RELATION TO HORMONE LEVELS

					During blood collection									
	Last behavioral test				Sexual behavior				Hormone response			Hormone ranking		
Rat No.	ML	IL	EL	PEI	ML	IL	EL	PEI	T	LH	Prl	T	LH	Prl
1	non-mater				non-mater				−	−	−	6	5	3
3	17.11	−	−	−	non-mater				−	−	−	2	5	1
6	non-mater				non-mater				−	−	+	5	5	2
7	0.26	0.66	14.1	4.55	−	7.0	19.4	8.3	+	+	+	3	1	6
8	non-mater				non-mater				−	−	+	4	3	5
15	15.67	15.80	35.13	7.40	non-mater				+	+	+	1	2	4

ML, IL, EL, PEI are latencies in min as previously defined. Last behavioral test before blood collection was carried out in May 1980. Hormone response is defined as at least 50% increase (+) in plasma T (testosterone), LH or Prl (prolactin) concentrations or less (−) in blood samples collected 5, 15, 30 or 60 min after entrance of a receptive female, as compared to basal hormonal levels. Hormone ranking is from 1 (highest) to 6 (lowest) in basal hormone concentrations.

Vol. 16, 1988

Vol. 16 1981

TABLE 3. HORMONAL RESPONSE TO RECEPTIVE FEMALE IN SEXUALLY EXPERIENCED MALE RATS IN THREE AGE GROUPS

Months	Hormone	Plasma hormone concentration (ng/ml ± SEM)				
		Basal	+5 (min after entrance of receptive female)	+15 (min after entrance of receptive female)	+30 (min after entrance of receptive female)	+60 (min after entrance of receptive female)
9	Testosterone	0.894 ± 0.079	1.192 ± 0.088[c]	1.273 ± 0.125[a]	1.685 ± 0.199[b]	1.966 ± 0.259[b]
14		1.405 ± 0.590	1.342 ± 0.469	1.484 ± 0.495	1.530 ± 0.147	1.911 ± 0.518
24		0.336 ± 0.017*	0.345 ± 0.069	0.384 ± 0.064	0.397 ± 0.088	0.436 ± 0.114
9	LH	14.0 ± 1.7	30.2 ± 4.5[b]	25.4 ± 3.2[b]	16.6 ± 2.5	12.5 ± 1.8
14		10.9 ± 0.6	15.9 ± 4.4	16.4 ± 3.5	10.8 ± 0.3	10.3
24		10.4 ± 0.1	13.5 ± 2.4	13.2 ± 2.1	11.9 ± 1.3	12.8 ± 2.5
9	Prolactin	40.1 ± 9.4	76.4 ± 15.3[a]	108.0 ± 14.8[c]	70.8 ± 22.1	29.7 ± 11.6
14		11.2 ± 1.1	18.6 ± 7.5	48.5 ± 12.5	33.1 ± 11.0	9.3 ± 1.3
24		40.6 ± 21.6	28.2 ± 9.2	39.5 ± 14.1	65.1 ± 39.0	58.6 ± 31.6

Animal numbers were 7, 3 and 6 respectively in 9-, 14- and 24-month old male rats. Blood was collected at times specified from jugular venous cannulas 3 days after surgical insertion. Basal blood collection was made 15 min before entrance of receptive females. All younger animals mated during the period when blood was collected; only one of 6 aged animals mated during the test. An asterisk indicates a significant difference ($p < 0.05$) between basal hormone levels in 9- and 24-month old groups. Within each age group, superscript "a" indicates a significant ($p < 0.05$) within-group comparison with basal hormone levels within that age group, "b," $p < 0.01$; "c" $p < 0.001$.

on sexual behavior (note increase in latencies in 9-month old rats, Table 1) and on hormonal levels (note LH in Table 3). The depression of LH was previously noted in this mating model (Kamel and Frankel, 1978), and is likely more severe in senescent male rats.

The advantage of the cannulated mating model is shown most clearly in the hormonal responses of animals #7 and #15. In #7, testosterone was maximal at 60 min, LH at 5 min, prolactin at 30 min; in #15, at 60, 15 and 15 min, respectively. These responses might have gone unnoticed in the previous blood-collection regimen (Frankel, 1981). Animal #7 mated consistently, both in the tests prior to blood collection and during blood collection, while animal #15 mated in both tests prior to but not during blood collection. The relationship of consistent mating and its respective hormonal response is highlighted in animal #3. In 3 tests prior to blood collection, he mated in the first two but not in the third, and failed to display a hormonal response to the receptive female during blood collection. Perhaps the hormonal response to a receptive female can be retained in a senescent non-mating male only when his sexual behavior has lapsed recently.

The relation of basal hormone concentration to sexual behavior is still in doubt. A decline in plasma testosterone concentration has always been associated with sexual impotency in aging human males (Talbert, 1977) and rats (Stone, 1938; Minnick *et al.*, 1946). Damassa *et al.* (1977), however, has recently shown that less than one-third normal levels of testosterone need be administered to castrated male rats to maintain normal sexuality. Further, there is no relationship between plasma testosterone concentrations and mating performance in mature male rats. Meanwhile, the possibility that high levels of plasma prolactin are associated with sexual dysfunction has been recently advanced by Bartke and associates (Svare *et al.*, 1979). Both viewpoints can be examined in Table 2, in which there is a suggestion that plasma testosterone and prolactin concentrations are related to sexual performance and associated hormonal responses. However, an examination of the numbers makes the conclusion doubtful. For example, the rankings in plasma testosterone concentrations are based on a range from 0.384 to 0.271 ng/ml, or 113 pg, a difference in hormone concentration which seems too subtle to be convincing. Prolactin concentrations are more sharply drawn, with their converse relationship to mating covering a range from 5.14 to 116.9 ng/ml. This hypothesis must be subjected to a study of large numbers of sexually active vs. sexually inactive aged male rats, in order to determine whether a change in a single or a combination of hormone concentrations is correlated to a decline in mating performance.

SUMMARY

A study was made of sexual behavior and the hormonal response to sexual behavior in 9-, 14- and 24-month old male rats, by collecting 5 successive blood samples in 75 min from indwelling jugular cannulas which permitted the rats to move freely. Only 1 of 6 aged rats mated during blood collection; plasma testosterone, LH and prolactin levels were elevated during mating in this rat, and during exposure to a receptive female in one other rat who had mated in an earlier test. All younger rats mated, and their hormone levels rose significantly during mating. Of basal concentrations of the 3 hormones, only plasma testosterone was significantly different (lower) in aged rats.

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Vol.
16
1981

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EFFECTS OF AGE AND SEX OF HOST MICE ON GROWTH AND DIFFERENTIATION OF TERATOCARCINOMA OTT6050

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Vol.
16
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INTRODUCTION

THE RELATIONSHIP between aging and carcinogenesis has been widely studied upon various hypotheses. However, after a tumor has been produced in the body, there are very few available studies of the growth and histological findings of the tumor and their correlation to host aging and sex. Since cancer is considered to be primarily a disease of middle and old age, host aging and sex are very important factors in understanding the host-tumor relationship, and for the management of cancer patient.

The murine teratocarcinoma has been widely used to study cellular differentiation and neoplastic growth. In the ascitic fluid of mice, these tumor cells form aggregates resembling 5 day stages of early mouse embryogenesis and are therefore termed embryoid bodies (Stevens, 1960). The solid tumors, produced by subcutaneous injection of embryoid bodies into mice, can differentiate to wide a range of tissue types, and also contain undifferentiated embryonal carcinoma cells (Pierce, 1960). Ability of differentiation and sensitivity to tumor environments of hosts are major characteristics of teratocarcinoma (Pierce, 1967). Teratocarcinoma is considered to be a good model to study the host-tumor relationship. In this report the effects of host aging and sex on the growth rate and differentiation of teratocarcinoma are described and a possible mechanism is discussed.

METHODS AND PROCEDURE

Tumor

Mouse teratocarcinoma OTT6050 was used in this study. The tumor originated from a six-day-old 129/Sv strain mouse embryo that had been grafted into the testis of a F1 hybrid mouse (Stevens, 1970). Embryoid bodies were maintained by intraperitoneal transplantation into 129/Sv mouse.

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Experimental animals

Ninety-one inbred 129/Sv strain mice from 2 to 70 weeks old were used in the experiments. Body weight against age of 300 healthy stocked 129/Sv mice were recorded to draw the developmental standard body weight curve against age under the conventional experimental conditions. All mice used for the experiments were within a standard deviation of the mean body weight. Tumor and mice were kindly supplied by Dr. Kazuko Uno of the department of Zoology Faculty of Science, Kyoto University.

Cell preparation

Embryoid bodies (EB) were harvested from mouse peritoneal cavity, and washed twice by centrifugation at 300 r.p.m. for 3 min. with Dulbecco's modification of Eagles medium (Flow Laboratories, U.S.A.). The EB were suspended in the medium above mentioned. Aliquot of EB suspension was taken to determine cell density following the method of Tsubouchi and Matsuzawa (Tsubouchi, 1973). After the EB suspension was incubated in 30% citric acid, nuclei stained with 0.1% crystal violet were counted with a hemocytometer.

Tumor inoculation

EB suspension 0.1 ml of a density of 8×10^7 cells/ml was subcutaneously inoculated to the back of 129/Sv mice (8×10^6 cells/mouse).

Measurement of tumor growth

Solid teratocarcinomas produced on the back of mice were measured by vernier calipers. The product of three principal diameters of the tumor was designated as Tumor Volume. A calibration curve was constructed in which the estimation of the tumor volume *in vivo* was plotted against actual volume measured after excision with measuring cylinder filled with water. Then the tumor growth curves were plotted on log-linear graph papers. The volume doubling times were calculated at each point, and these data were fitted to the doubling time curve of the Gompertzian curve with a least-square method (McCredie, 1965); the parameters of the Gompertzian curve A and B were then determined to compare the growth rates of teratocarcinomas at each host age. Survival times of mice where Tumor Volume exceeded a hundred (products of three diameters of the tumor exceed 100, 5mm × 5mm × 4mm, for example, designated on Day 0), to the death of the mice were also studied. At death, autopsy was carried out and the excised tumor was fixed, sectioned, and stained with hematoxylin and eosin for histological examination.

RESULTS

Standard body weight curve

In our experimental conditions, 129/Sv mice growth-spurted at 2 to 6 weeks old, matured sexually at 8 to 10 weeks old. They grew fat at 30 weeks, but very old mice become thin at 70 weeks (Fig. 1). The maximum life span reached two years.

Result of inoculation

Tumor subcutaneously inoculated took successfully in 69 of 91 mice used. All tumors of 65 mice were measured and plotted for, the tumor growth curve. Table 1 showed their age distribution. Others had visceral metastases or died of infection or accident.

Calibration curve

Figure 2 showed that there was a linear correlation between the estimated tumor volume in vivo and the actual excised tumor volume by measuring cylinder. Therefore it was considered that there was no systematic error in using the product of three diameters as the tumor volume, and it was plotted to construct the tumor growth curve.

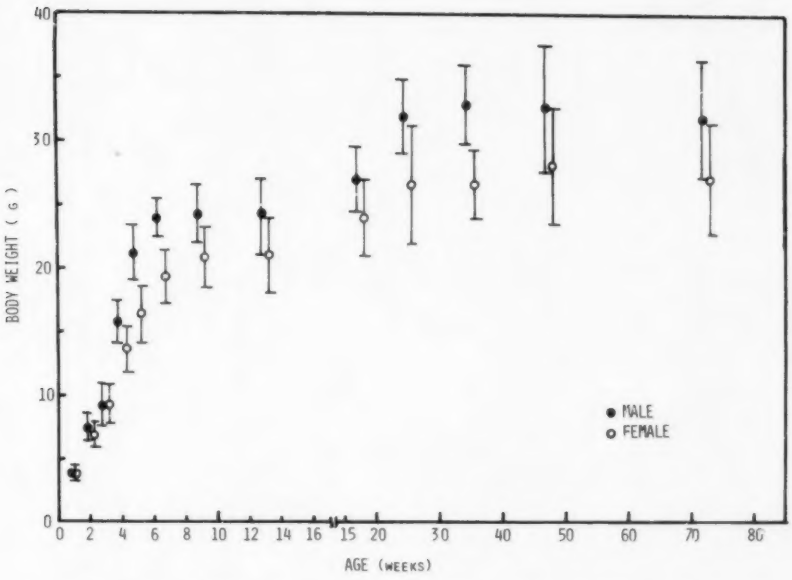


FIG. 1. Developmental standard body weight curve of 129/Sv mice. Abscissa: Age (weeks) of 129/Sv mice; Ordinate: Mice body weight (grams) mean and a standard deviation, (•) male; (o) female.

Growth curve

The data of tumor growth curves of both male and female mice of 2, 3, 10, 20–30, 50, and 70 weeks old were plotted on log-linear graphs (Fig. 3). In the 2 and 3 weeks old both male and female, tumors grew fastest. In the male mice from 10 to 70 weeks old, tumors grew slower than in 2 and 3 weeks old mice. But during these period in males there were no significant differences in tumor growth rate. On the other hand, in the female mice, tumor growth rate became slow from 10 to 70 weeks old in that order. Next, the tumor volume data for 3 to 5 days were averaged in each age and were plotted to compare on the same graph (Fig. 4). Figure 4 showed that in the male, tumor growth curves were apparently divided into two groups, 2 to 3 weeks old, and 10 to 70 weeks old. In contrast, in the female they were divided into three groups, 2 to 3 weeks old, 10 to 30 weeks old and 50 to 70 weeks old. In other words, tumor growth rate of teratocarcinoma got slower by two stages in male, and three stages in female as host mice grew older.

TABLE 1. NUMBER OF MICE USED FOR TUMOR GROWTH CURVE

Age (weeks)	Male	Female	Total
2	6	4	10
3	4	5	9
10	8	7	15
20–30	4	4	8
50	8	8	16
70	4	3	7
Total	34	31	65

Vol. 16 1981

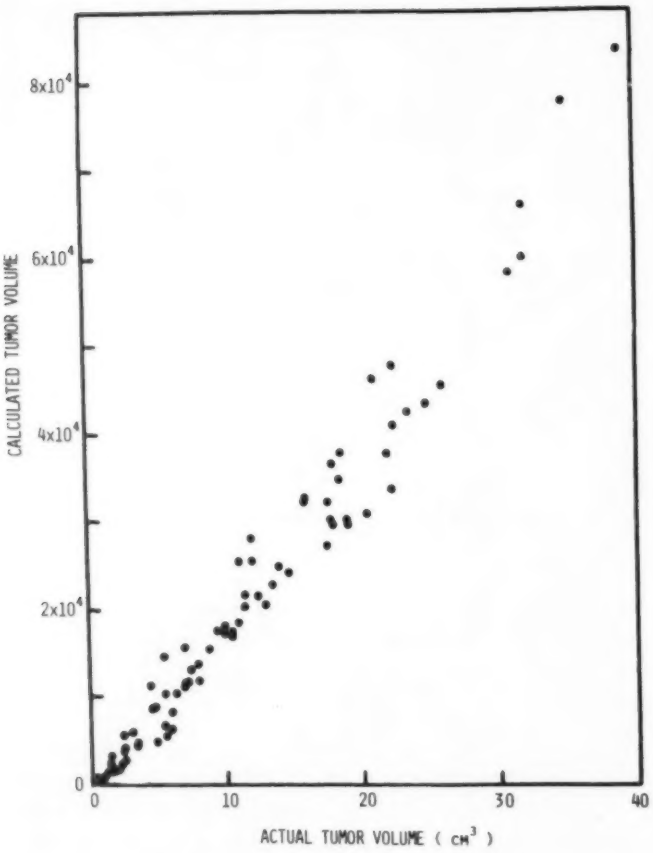


FIG. 2. Tumor volume calibration curve. Abscissa: Excised tumor volume (cm^2) measured by measuring cylinder filled with water; Ordinate: Estimated tumor volume that is the product of three principal diameters of the tumor on the back of mice measured by vernier calipers.

Volume doubling time of the tumors in each age were calculated. As the tumor doubling times were not constant but became prolonged with tumor growth, they were plotted against measured tumor volume (Fig. 5). In the male, at the Tumor Volume 500, doubling time was 2.1 days for 2 to 3 weeks, 3.0 days for 10 to 30 weeks, 2.9 days for 50- to 70-week-old mice. Tumor doubling time in the juvenile differs by 0.9 days from that of adult. In the female at the Tumor Volume 500, doubling time was 2.2 days for 2 to 3 weeks, 3.2 days for 10 to 30 weeks, 4 days for 50- to 70-week-old mice. When the tumor grew large, the doubling time got long and reached about 7 days and the influence of host age and sex on the doubling time became unclear. In other words, when tumor grew fastest, differences of the tumor growth rate by host age were largest.

The analysis of tumor growth curves by fitting the Gompertzian equation (1) to experimental data was carried out.

$$V = V_0 e^{\frac{A}{B}(1-e^{-Bt})} \tag{1}$$

Vol. 16 1981

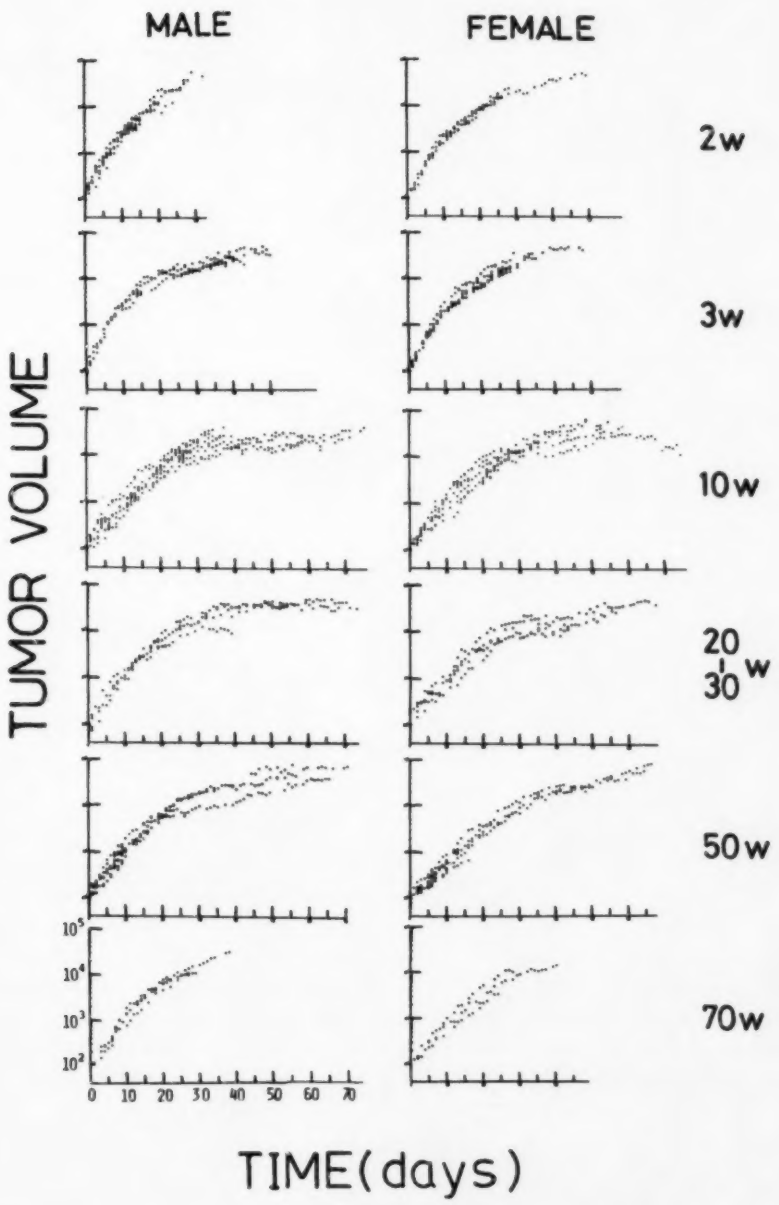


FIG. 3. Tumor growth curves in each age and sex hosts mice. Solid teratocarcinomas inoculated on the back of the 129/Sv mice of 2, 3, 10, 20–30, 50, and 70 weeks old (at inoculation) male (left side) and female (right) were measured and plotted. Abscissa: Time (days) after the tumor volume exceeded 100; Ordinate: Tumor volume plotted on log scale (see in the text).

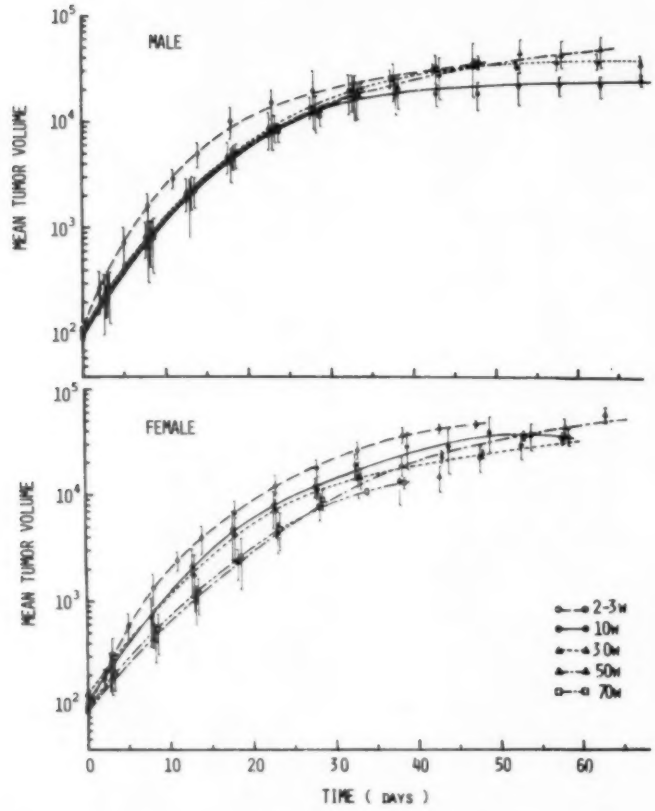


FIG. 4. Average tumor growth curve, male (top) and female (bottom), o-----o 2 and 3 weeks, •——• 10 weeks, ▲-----▲ 30 weeks, △-----△ 50 weeks, □-----□ 70-week-old host mice.

This equation has two constants, A is related to the initial growth and B is retarding fraction factor (Steel, 1977). Its applicability to various experimental tumors was demonstrated by Laird (1964), and McCredie (1965). The doubling time and time from the Day 0 were calculated (Fig. 6). The doubling time curves from the Day 0 to the Day 30 were fitted to the doubling time equation of Gompertzian curve (2) by least squares method.

$$T\,\text{doubling} = \frac{InB}{A} e^{Bt} \tag{2}$$

Then, both constants A and B were plotted against host age (Fig. 7). Both coefficient A and B in male and in female fell sharply with advancing age from 2 to 10 weeks old. In the male both A and B were unchanged with advancing age from 10 to 70 weeks old. But coefficient A fell faster than B in the female from 10 to 50 weeks old. There were no significant changes in A and B between 50 to 70 week old female. Thus the changes of tumor growth during hosts aging were described through the changes of coefficient A and B, growth factor and retardation factor.

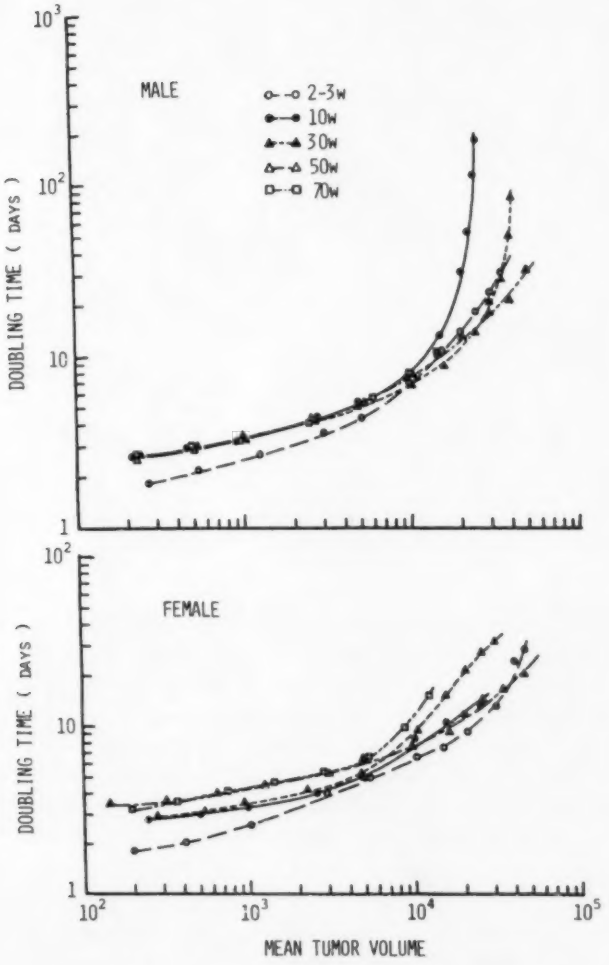


FIG. 5. Doubling time curves of teratocarcinoma. Male (top) and female (bottom). Abscissa: Mean tumor volume, log scale; Ordinate: Volume doubling time (days) of tumor, log scale. Symbols are the same as Fig. 4.

Histological examination

Solid teratocarcinoma, produced by subcutaneous transplantation, consisted of various kinds of tissues, well differentiated and undifferentiated. The tissue types of differentiation are shown in Figs. 8a-f. Tumors contained very large necrotic areas in the juvenile hosts 2 and 3 weeks old, when tumors grew at maximum speed. Most of the residual viable tissues consisted of undifferentiated carcinoma cells, and there were only a few clumps of well differentiated tissues, nervous tissue and ciliated or nonciliated epithelium. On the adult and middle aged mice from 10 to 50 weeks old, tumor also contained necrotic areas, but there were large masses of well differentiated kinds of tissues around the necrosis. These included nervous tissues that are recognized by the presence of primitive neural

Vol. 16 1981

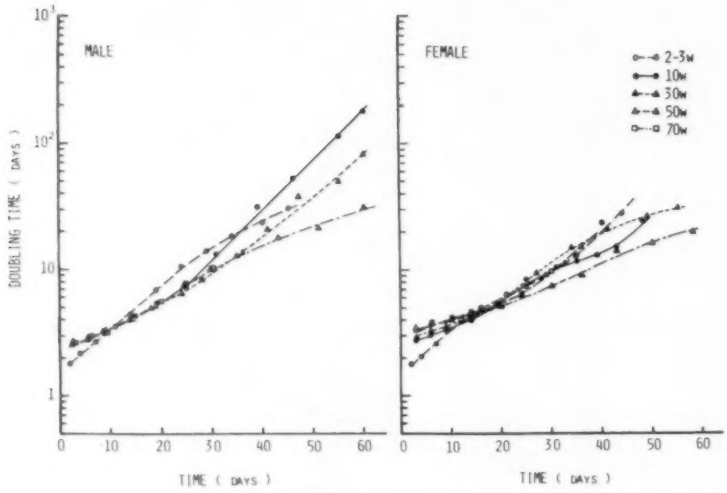


FIG. 6. Volume doubling time and day. Abscissa: Time (days) from tumor volume exceeded 100; Ordinate: Volume doubling time of tumor (days).

tubules, epithelial tissues ciliated, nonciliated or keratinized, with which cysts and tubules were often lined, and also included keratin pearls. Fatty tissues, hyaline cartilage, bone, and skeletal muscle were also found. Only small clumps of embryonal carcinoma cells were observed among the differentiated tissues and in the peripheral area of the tumors. On the 70-week-old mice, tumors contained larger necrotic tissues than that of 50-week-old mice, less embryonal carcinoma cells than that of 2- and 3-week-old mice, a few types of differentiated tissues of nervous tissue and nonciliated epithelium. There was no significant differences in the histological types of tumors by hosts' sex.

Survival days

It takes 21.5 ± 4.7 days for the tumor to grow up to the Tumor Volume 100 the Day 0, after inoculation (latent times). There was a positive correlation between the survival days of host mice and the age of the mice ($r = 0.680$, $p < 0.001$). Figure 9 showed that older mice could survive longer. But 70-week-old mice that died of old age, were excluded. Figure 10 showed that the longer the mice survived, the bigger the tumors on the mice grew ($r = 0.813$, $p < 0.001$).

DISCUSSION

It has been generally believed by physicians that tumors advance very fast and are usually highly malignant in the young patient, but tumors often progress slowly and the older patient could survive long. But this impression was based on insufficient evidence. Tumor incidence or transplantability of experimental tumor and aging have already been studied by some authors (Cook, 1964; Kerkvliet, 1973). But very few data is available about the tumor growth rate. It was preliminarily shown that in rats, Morris hepatoma, solid tumors transplanted subcutaneously with trocar, grew slower in aged rats than

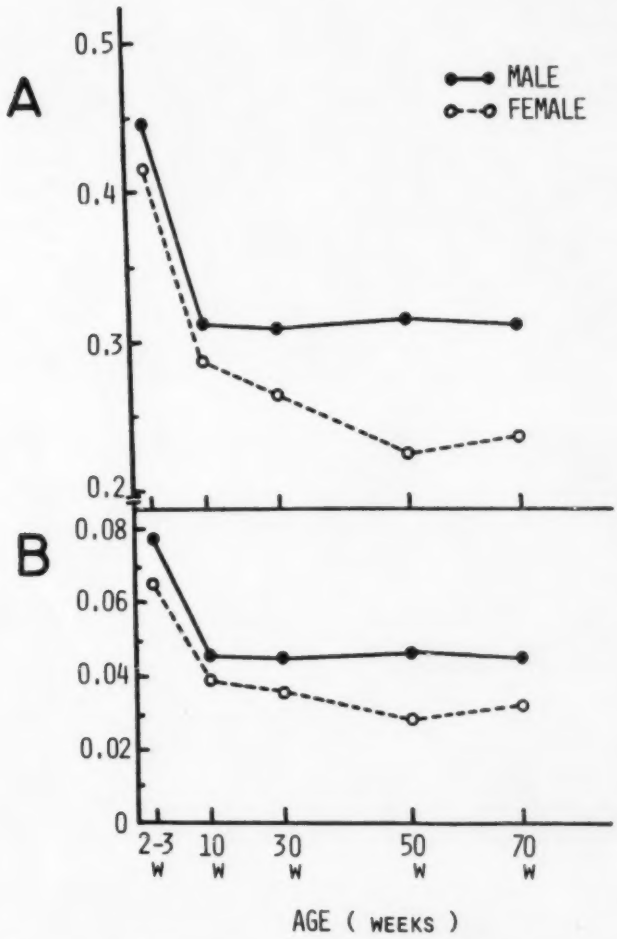
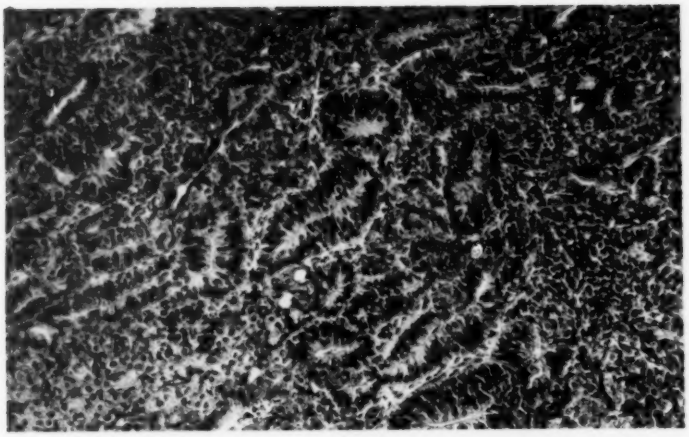


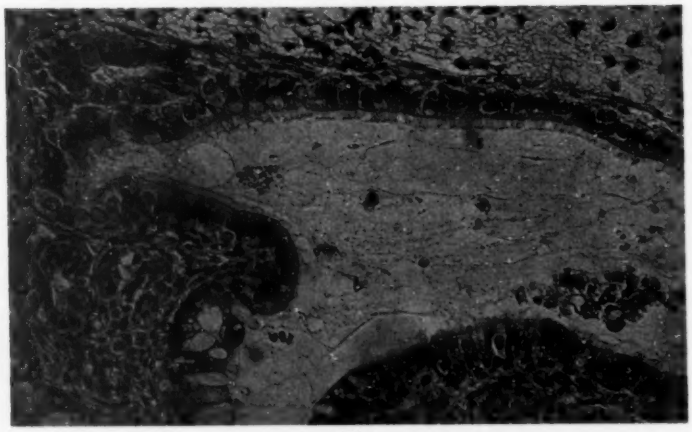
FIG. 7. Changes of constants *A* and *B* of the Gompertzian equation with which tumor growth curves of hosts of each age and sex were fitted. Abscissa: Age (weeks old) of host mice. Constants *A* (top) and *B* (bottom), •——• male, and o— — —o female.

young rats (Morris, 1966). The differences from the literature and our experiment lies its design, completeness of data and type of tumor. We measured large numbers of tumors, on the basis of equal numbers of cells inoculated, using nuclei counting method, and analyzed the tumor growth curves with the Gompertzian equation. Teratocarcinoma and syngenic hosts were used and the tumor differentiation pattern was observed (Kubota, 1980a, 1980b).

In this experiment, on the juvenile hosts (2 and 3 w.o.) inoculated just after weaning, teratocarcinoma grew fastest and consisted of undifferentiated tissues. In the young adult mice (10 w.o.) tumor growth slowed down and the tumors contained various kinds of well differentiated tissues around the necrotic area. Thus it might be considered that there is an



(a)



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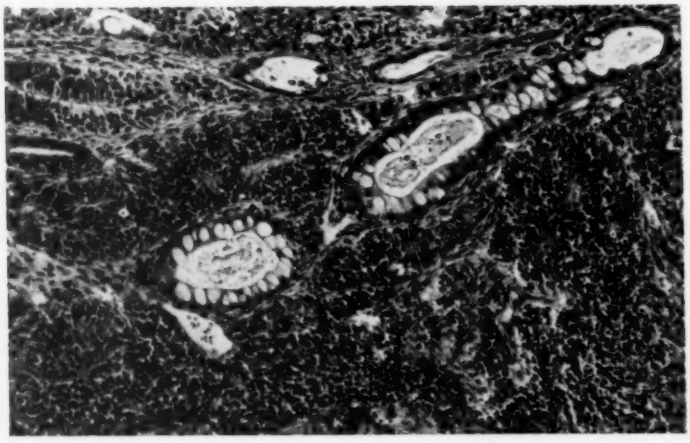
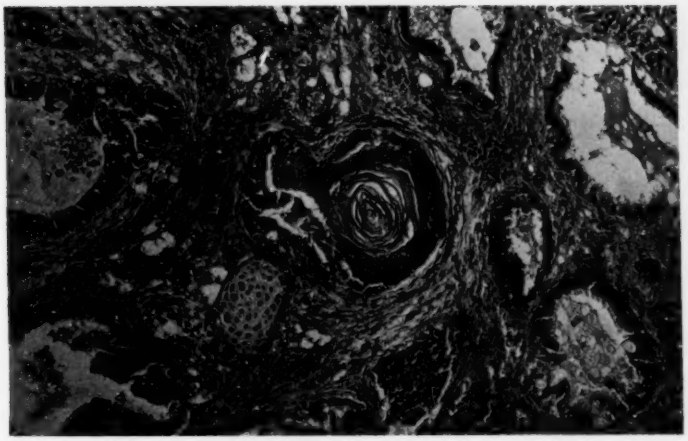


(c)

FIGS. 8a-c. Photomicrographs of teratocarcinoma inoculated on the backs of 10-week-old 129/Sv mice. (a) Neural tubules, (b) ciliated epithelium, and (c) mucus secreting epithelium. (a, c × 200; b × 400)

Vol.
16
1981



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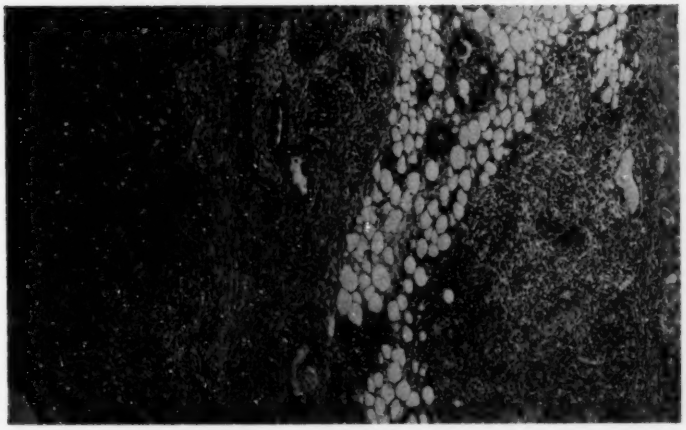

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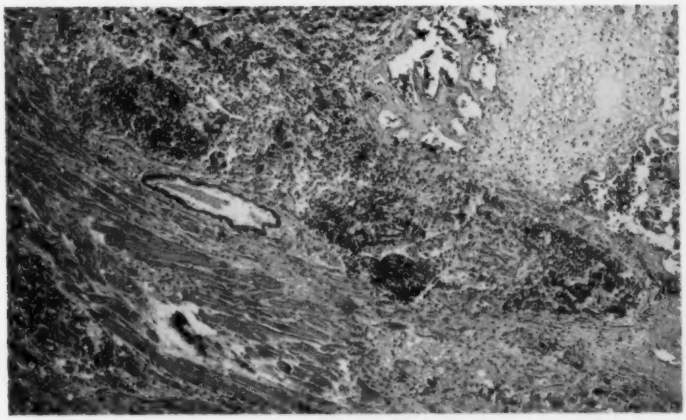

(f)

FIGS. 8d-f. Photomicrographs of teratocarcinoma inoculated on the backs of 10-week-old 129/Sv mice. (d) keratim pearl and cartilage, (e) undifferentiated carcinoma cells and fatty tissue, (f) cartilage, bone and skeletal muscle. (d × 200, e, f × 100)

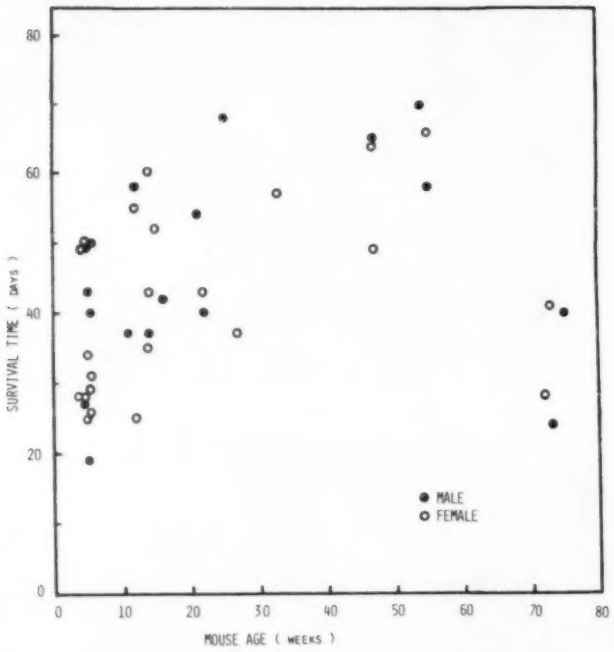

FIG. 9. Survival time (days after the tumor volume exceeded 100) and mouse age (weeks at inoculation).

inverse relationship between tumor growth and differentiation. Tumor growth was constant in the male from 10 to 70 w.o. But in the female, tumor growth slowed down with advancing age from 10 to 70 w.o. In the 70 w.o. mice tumors contained large necrotic areas and less embryonal carcinoma cells and few differentiated tissues.

Tumor growth analysis is a basic and important method that can be applied to human cancer in the study of tumor nature (Charbit, 1971) and assessment of treatment (Kubota, 1980c). The Gompertzian equation has been generally used for simulating tumor growth data (Winsor, 1932; Laird, 1964). The parameter A is called the growth constant of the initial limiting exponential, and the constant B determines the rate of increase of doubling time, called retardation constant (Steel, 1977). At 2 and 3 w.o. both constant A and B were largest. Tumor growth seemed to be accelerated when the juvenile host mice grew fast, and growth constant A become largest. When the tumor growth was accelerated, the discrepancy of growth rate between the tumor and its substrate, mainly blood vessels, seemed to be increased. The necrotic area was expanded in proportion to the discrepancy (Tannock, 1968). Thus the retardation constant B seemed to be large in rapid growing tumors. From 3 to 10 w.o., both A and B fell rapidly. In the male, A and B did not change from 10 to 70 w.o.. But in the female, from 10 to 70 w.o., parameter A decreased greater than the parameter B. As the changes of retardation constant B was small, it might be suggested that the delay of tumor growth in the female did not depend on the cell loss of tumors, but on the growth fraction or cell cycle. In addition to this, the cell loss measured preliminarily by ^{125}I-deoxyuridine method (Dethlefsen, 1971; Hotter, 1969) showed no significant differences between the tumors of 10- and 30-week-old females (data was not

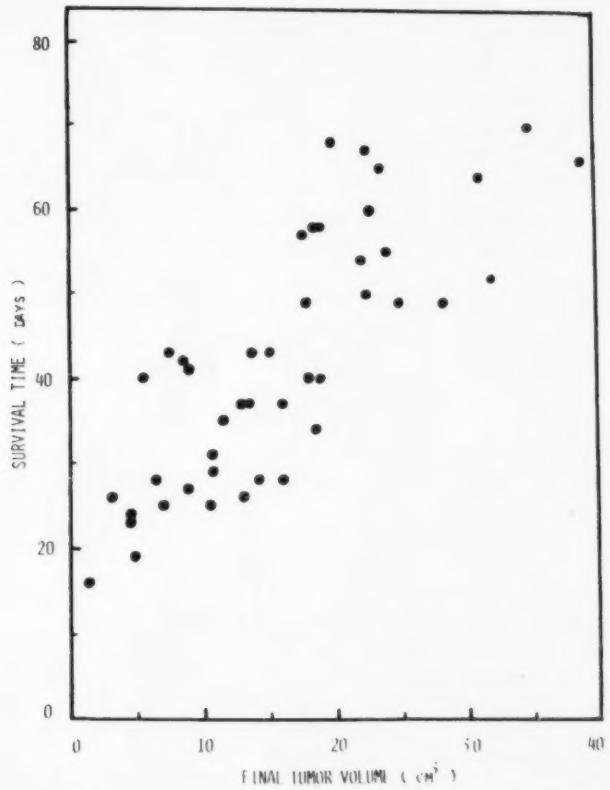

FIG. 10. Survival time (days) and excised tumor volume (cm^3) at the autopsy.

shown). Appearance of large necrotic area in 70 w.o. might be explained by the delay of neovascularization with host aging (Yamaura, 1980).

Secretion of various kinds of hormones and growth factors (Florini, 1980) have been known to change with aging. If the teratocarcinoma tumor growth was to be stimulated by estrogen, the differences by host sex would be explained easily. It was preliminarily observed that the growth rate of teratocarcinoma in 50 w.o. female mice, injected with estradiol intraperitonealy, was as fast as that in 10 w.o. female mice. The possibility of growth stimulation by estrogen and growth inhibition by an antiestrogen agent are suggested.

Further experiments of growth kinetic study and host aging in relation to estrogen are now being undertaken.

SUMMARY

The effect of age and sex of host mice 129/Sv on the growth rate and differentiation of teratocarcinoma OTT6050 was studied. Solid teratocarcinoma, inoculated on the back of mice, was measured by vernier calipers. Tumor volume was estimated from the product of three principal diameters. After drawing the calibration curve, the tumor growth curves

Vol. 16 1981

of both male and female mice from 2 to 70 weeks old were plotted, compared, and analyzed by fitting the Gompertzian equation. On the juvenile hosts (2 and 3 w.o.), inoculated just after weaning, tumors grew fastest and consisted of necrosis and undifferentiated tissues. In the young adult mice (10 w.o.) tumor growth slowed down and tumors contained various kinds of well differentiated tissues around the necrotic area. Tumor growth was constant in the male from 10 to 70 w.o. But in the female, tumor growth slowed down with advancing age, from 10 to 70 w.o. In the 70 w.o. mice tumors contained large necrotic areas and less embryonal carcinoma cells and few differentiated tissues. There was no histological differences of tumor by host sex. Survival data showed that older mice could survive longer, with the exception of 70 w.o. mice. Teratocarcinoma growth curves showed the inhibiting effect of host aging on tumor growth rate, and the different tumor growth rate by host sex suggested the possible growth stimulation by estrogen.

Acknowledgment—We would like to thank Dr. Harutsugu Yamaura and Dr. Takehito Sasaki for their thoughtful discussion and suggestions, and Mr. Yasuhiro Sugawara and Mr. Tachio Sato for their excellent technical assistance.

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AGE RELATED CHANGES IN MUSCLE CONNECTIVE TISSUE: ACID MUCOPOLYSACCHARIDES AND STRUCTURAL GLYCOPROTEIN

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Vol.
16
1981

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INTRODUCTION

IT HAS BEEN shown that the pattern of age related changes with mucopolysaccharides and neutral structural glycoproteins varies with tissues and animals (Sobel *et al.*, 1970; Fleishmajer *et al.*, 1972; Hjerquist and Lemperg, 1972; Sames *et al.*, 1974; Kohn, 1977). However, there is no report on acid mucopolysaccharide (AMPS) and structural glycoprotein content in different muscles as a function of age. This paper describes the functional significance of AMPS to collagen ratio during ageing in slow, fast and cardiac muscles of rat.

MATERIALS AND METHODS

AMPS content was estimated in terms of its constituents, uronic acid and hexosamines. Soleus (slow contracting, red muscle), Extensor digitorum longus (fast contracting, white muscle) and cardiac muscles of 5, 10, 15, 20 and 25-month-old rats were removed, dried and weighed. For estimation of uronic acid, AMPS was extracted from 20–30 mg dry muscle tissue in 0.5N NaOH according to the method of Clausen (1962) and was determined by Dische's (1947) carbazole method. In the case of hexosamines, 20 mg dry muscle tissue was hydrolysed in 4N HCl for 4 hours in a boiling water bath and was estimated according to the method of Boas (1953). D. glucuronic acid and N acetyl glucosamine were used as standards.

β-glucuronidase and β-N acetyl glucosaminidase activity

The weighed muscles were homogenised in ice cold distilled water and centrifuged at 3000g for 10 minutes at 0–4°C. The activities of β-glucuronidase and β-N acetyl glucosaminidase of the above extract were determined according to Levy and Marsh (1959), and Weissman *et al.* (1964) using phenolphthalein glucuronidate and b-phenyl N acetyl glucosaminide as substrates respectively.

Structural glycoprotein

Structural glycoprotein was extracted from the muscles according to Robert and Robert (1974) and estimated interms of total hexoses according to Dubois *et al.* (1956) using D-galactose as a standard.

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RESULTS AND DISCUSSION

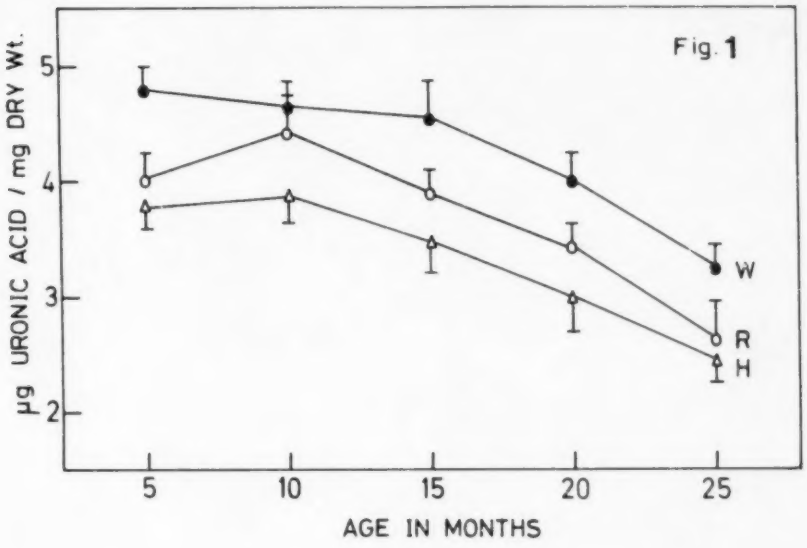
The present results with reference to uronic acid and hexosamines indicate a decrease in AMPS content with advance in age in all the three muscles. The level of hexosamine indicates the tissue content of AMPS as well as structural glycoprotein while that of uronic acid refers to only AMPS. Uronic acid shows a sharp decline in all the three muscles between 10 and 25 months of age (Fig. 1). This would lead to a decrease in water binding capacity in the extracellular space of the muscle. Total hexosamine content shows an initial increase up to 10 months in cardiac and up to 15 months in red and white muscle and thereafter decreases (Fig. 2). As hexosamine is also a constituent of glycoprotein, the initial increase in hexosamine may be due to increase in structural glycoprotein content in these muscles. But with further advance in age, AMPS may decrease to a greater extent resulting in the decrease of total hexosamine level.

Activities of β-glucuronidase and β-N acetyl glucosaminidase, the lysosomal enzymes involved in the breakdown of collagen-AMPS complex are taken as indices to measure the catabolic process of AMPS. β-glucuronidase activity increases from 101–132 units in red, 95–125 units in white and 110–148 units in cardiac muscle between 10 and 15 months of age and is more or less constant thereafter in red and white while in cardiac muscle there is an increase even after 15 months (Fig. 3). The activity of β-N acetyl glucosaminidase increases by 38, 30 and 22% in red, white and cardiac muscle as the age advances from 5 to 25 months (Fig. 4). Increase in the activities of these enzymes may be one of the factors responsible for the decrease in AMPS content.

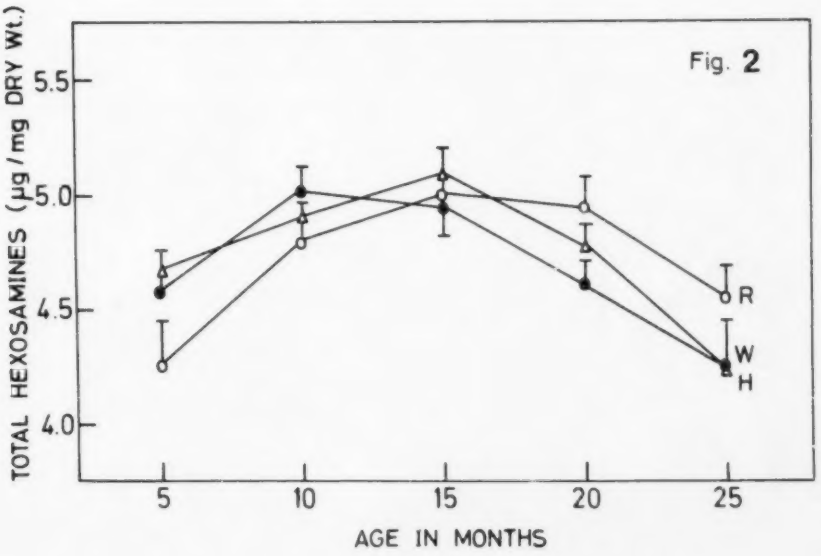
Activity of β-glucuronidase and β-N acetyl glucosaminidase have been shown to increase by 23% and 19% respectively in the skeletal muscle of mice as the age increased from 4 to 7 months (Pilstrom *et al.*, 1978). Wildenthal *et al.* (1977) have reported that the activity of β-N acetyl glucosaminidase decreased by 17% in the left ventricle of rats (1–2 months vs 10–14 months) while the activity remained unchanged in rabbits (1½–3 months vs 6–12 months). Total activities of β-N acetyl glucosaminidase in the lysosomal and microsomal fractions of heart muscle have been shown to decrease slightly with age (Asano *et al.*, 1979). The present results and that of others show heterogenous changes in the activity of lysosomal enzymes during ageing with reference to different organ systems and species studied.

With advance in age when AMPS content decreases, collagen content increases (Mohan and Radha, 1980), and thereby shows a marked decrease in the ratio of AMPS to collagen (gel-fiber) which is reflected in the ratios of uronic acid to hydroxyproline and hexosamine to hydroxyproline (Figs. 5 & 6). AMPS to collagen ratio is also known to decrease with age for rat kidney (Ber *et al.*, 1969), human skin, aorta and myocardium (Clausen, 1962) and this decrease in the ratio can be counted as one of the characteristics of age which would result in impaired supply of oxygen to the cells (Sobel, 1967).

The structural glycoprotein content expressed in terms of total hexoses shows an age related increase in red and cardiac muscles, while in white muscle it increases up to 15 months and remains more or less constant thereafter (Fig. 7). In contrast to AMPS to collagen ratio, the glycoprotein to collagen ratio does not show significant change with age in all the three muscles (Table 1). The increase in the structural glycoprotein content suggests its role in the organization of collagen net work (Robert and Robert, 1974). Buddette and Kichtenstein (1963) found an increase in the concentration of glycoprotein with age in the lung while Hakomori (1963) reported a decrease in skin and aorta.



Vol.
16
1981



FIGS. 1 and 2. Acid Mucopolysaccharide (AMPS) content in terms of its constitutents, uronic acid (Fig. 1) and hexosamine (Fig. 2). (R = red muscle, W = white muscle, H = cardiac muscle)

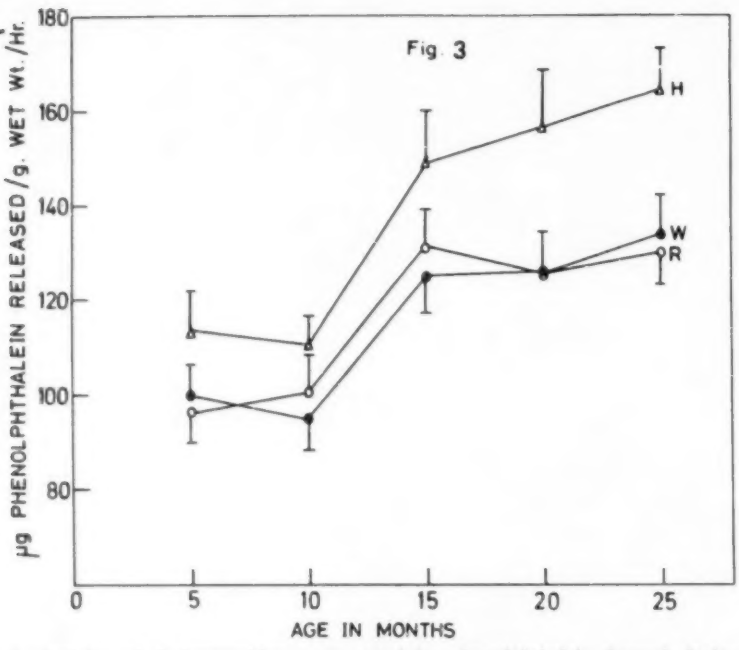


FIG. 3. Activity of β-glucuronidase represented by phenolphthalein formed during the reaction. (R = red muscle, W = white muscle, H = cardiac muscle)

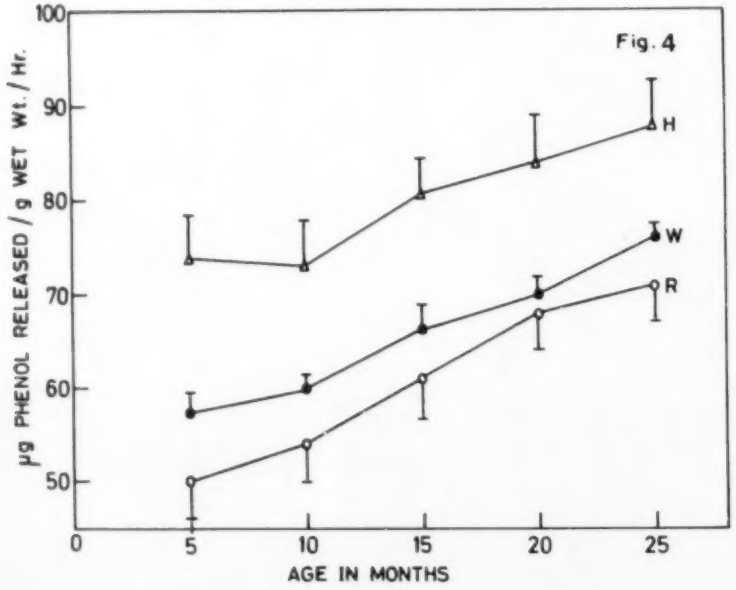
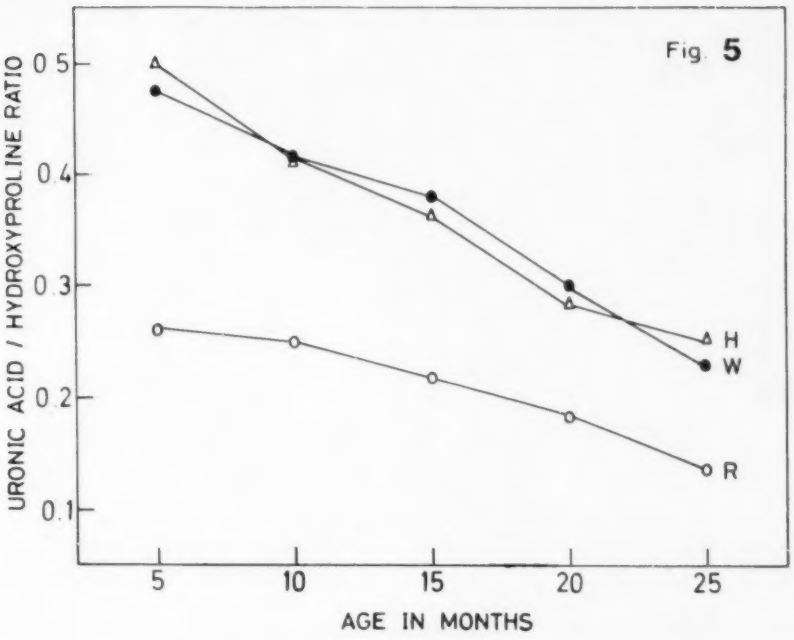
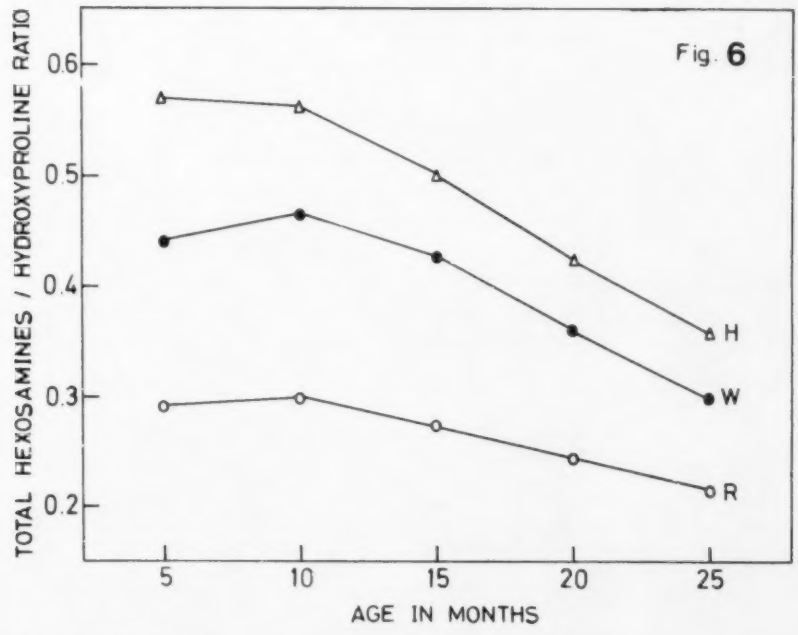


FIG. 4. Activity of β-Nacetyl glucosaminidase represented by phenol formed during the reaction.





FIGS. 5 and 6. AMPS to Collagen ratio represented by their constituents: Uronic acid/ Hydroxyproline (Fig. 5), Hexosamine/Hydroxyproline (Fig. 6). (R = red muscle, W = white muscle, H = cardiac muscle)

Vol. 16 1981

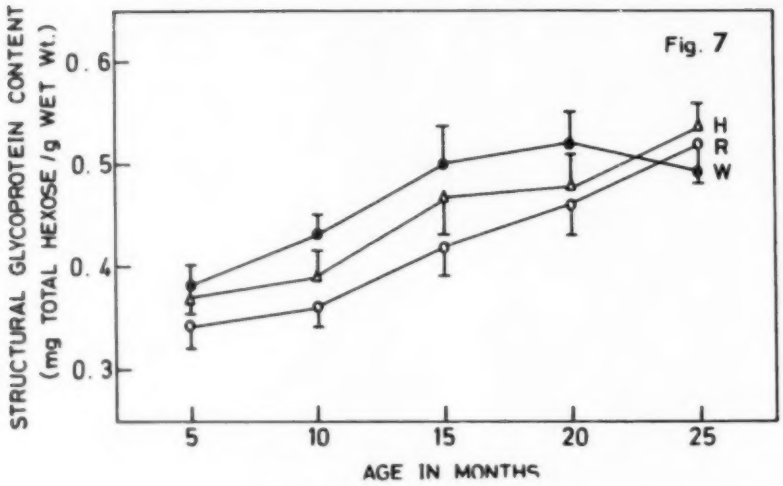


FIG. 7. Structural glycoprotein content of the muscle estimated in terms of its constitutent, total hexose. Values are represented as mean ± S.D. of 5 observations. (R = red muscle, W = white muscle, H = cardiac muscle)

Schematic representation of the possible age changes in muscle connective tissue leading to the impairment in muscle function is summarized in Fig. 8. Our results showing marked decrease in AMPS content, AMPS to collagen ratio, increase in insoluble, total collagen (Mohan and Radha, 1980), structural glycoprotein and protein degradation rate (Mohan and Radha, 1978) in different muscles would throw some light on the factors which affect the contractility and diffusion processes as the age advances.

SUMMARY

Age related changes in AMPS (uronic acid and hexosamine), structural glycoprotein, β-glucuronidase and β-N acetyl glucosaminidase were studied in red, white and cardiac muscles of 5, 10, 15, 20 and 25-month-old rats. Uronic acid and hexosamine content indicated a decrease in AMPS with advance in age in all the three muscles. β-glucuronidase activity increased from 101 to 132 units in red, 95 to 125 units in white and 110 to 148 units in cardiac muscle between 10 and 15 months of age while that of β-N acetyl glucosaminidase increased by 38, 30 and 22% in the three muscles respectively between 5 and 25 months of age. Structural glycoprotein showed an age related increase in red and cardiac

TABLE 1. RATIO OF STRUCTURAL GLYCOPROTEIN TO COLLAGEN (TOTAL HEXOSES/HYDROXYPROLINE) IN RED, WHITE AND CARDIAC MUSCLES OF RAT AS A FUNCTION OF AGE

	Age in months				
Muscle	5	10	15	20	25
Red	0.088	0.082	0.090	0.094	0.100
White	0.148	0.158	0.167	0.158	0.138
Cardiac	0.200	0.171	0.198	0.183	0.187

Values are represented as mean of 5 estimations.

Vol. 16 1981

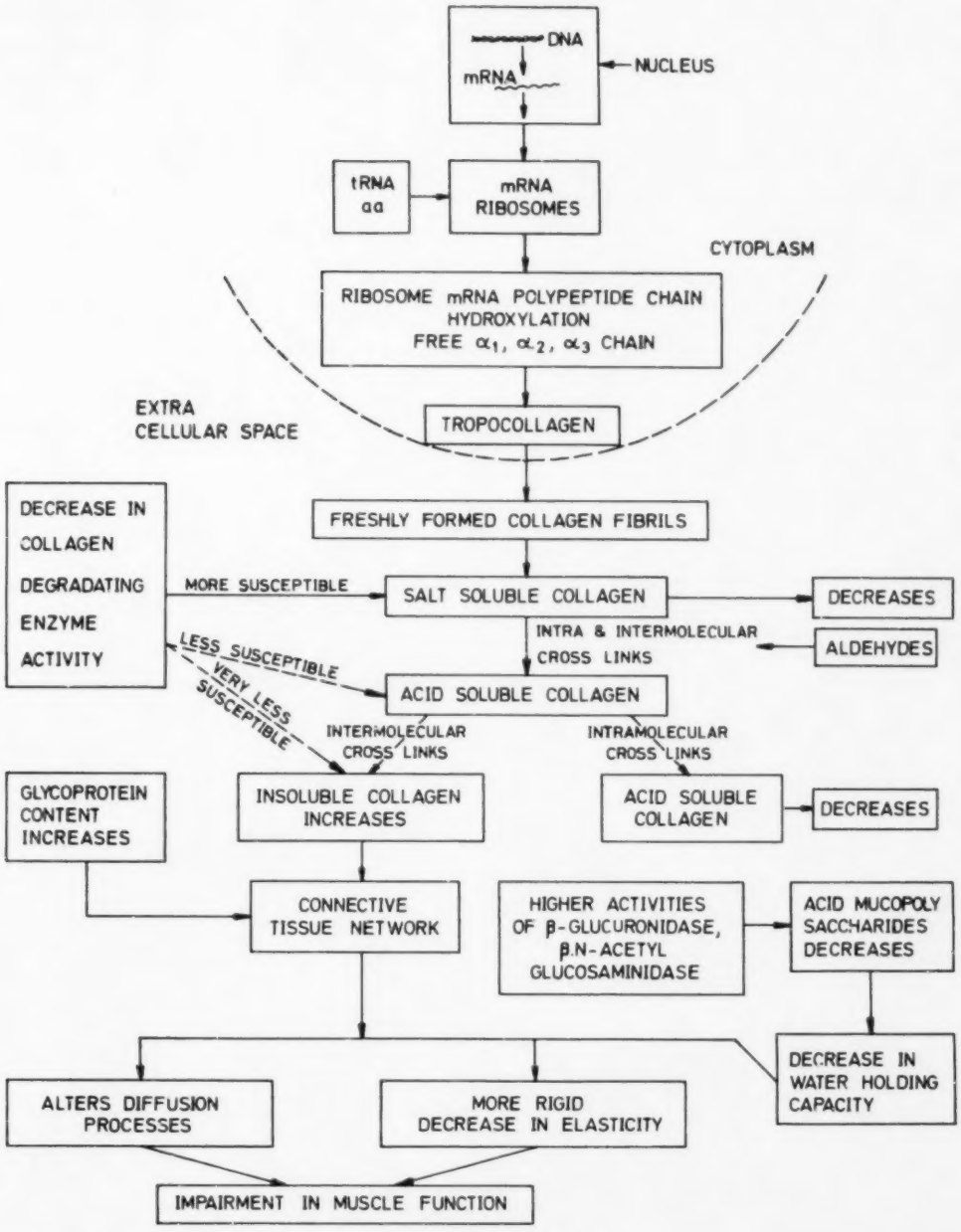


FIG. 8. Schematic representation of age changes in connective tissue leading to impairment in muscle function.

muscle while in white muscle it increased up to 15 months and remained more or less constant thereafter. Significance of these results in terms of the ratios of AMPS to collagen and their possible relevance in explaining the age related decline in muscle function are discussed.

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EFFICIENCY OF MATING IN C57BL/6J FEMALE MICE AS A FUNCTION OF AGE AND PREVIOUS PARITY

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Vol.
16
1981

Abstract—Age did not alter the incidence of insemination between the ages of 3 to 12 mo. in C57BL/6J female mice mated with C3HeB/FeJ males. Virgin females, 3–12 mo old, required a much longer mating period to achieve first insemination; the failure rate of pregnancy was not influenced by the interval between exposure to a male and insemination. Virgin mice which failed to become pregnant after the first insemination were shown to be fertile on second mating.

INTRODUCTION

DURING BREEDING studies, it is often observed that some mice which mate and which are inseminated by the criterion of a coital (vaginal or seminal) plug subsequently fail to become pregnant. For example, about 10% of previously parous C57BL/6J mice (3–12 mo) failed to show implantation sites or elevations of plasma progesterone as expected at 6–10 days after mating and insemination (Holinka *et al.*, 1979a). Since the efficiency of mating is usually not reported in studies of age-changes in pregnancy, it becomes important to know how representative those mice selected by their ability to become pregnant are of the total population. Mice with variations in reproductive performance are of interest in their own right, such as the small subgroups (6%) of older mice in which all fetuses are resorbed after gestation day 12 (Holinka *et al.*, 1979a; Gosden *et al.*, 1980): age changes in the frequency of such subgroups may give insight into endocrine and neural mechanisms of reproductive senescence. In this report, we analyze the breeding patterns in cohorts of C57BL/6J female mice of different ages and reproductive history over extended periods for alterations in the efficiency of mating and pregnancy at ages up to 12 mo, when most mice are still fertile (Holinka *et al.*, 1979a).

MATERIALS AND METHODS

Ten-week-old virgin female C57BL/6J mice (used in experimental Groups 1 to 5 and in Group 7; Table 1) and C3HeB/FeJ virgin males were purchased from the Jackson Laboratory, Bar Harbor, Maine. The C3HeB/FeJ males were chosen because this strain breeds more vigorously than C57BL/6J mice (Richardson, 1973). The

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TABLE 1. BREEDING PATTERNS OF C57BL/6J FEMALE MICE AT DIFFERENT AGES AND STATES OF PARITY

Experimental group	Cohort	State of parity at mating	Total # females mated	Age of females at mating	Season of mating	Maximum period kept with male	# Mice inseminated (%)	# Mice inseminated without subsequent pregnancy (%)
1	1024	Virgin	69	3.5 mo.	October	13 days	23 (33%)	4 (17.4%)
2a	1025	Virgin	235	3–5 mo.	February to April	7 weeks	218 (93%)	43 (19.7%)
2b		Second mating of inseminated, nonpregnant females of Group 2a	40			-a	40b (100%)	4c (10.0%)
3	1015	One previous pregnancy	30	8 mo.	July	6 days	27 (90%)	4 (14.8%)
4	1015	Two previous pregnancies (same mice as Group 3)	29	12 mo.	November	18 days	24 (83%)	7 (29.1%)
5	1015	Virgin; same cohort as Groups 3 and 4	27	12 mo.	November	18 days	16 (59%)	5 (31.3%)
6	1018	Retired breeders	27	11.5 mo.	July	19 days	22 (81%)	-d
7	1024 1025 1029	One previous pregnancy	370	3–7 mo.	January to May	7 weeks	319 (86%)	21/225e (9.3%)
8	1026 1028 1030 1032	Retired breeders	465	11–12 mo.	March to May	25 days	433 (93%)	36/320e (11.3%)

[a]Data were not extracted from records.

[b]Three of the 43 inseminated nonpregnant mice of group 2a were not remated.

[c]Two of these four animals were mated for a third time; both became pregnant.

[d]The Breeding data of Group 6 were obtained from a pilot study to determine the stress effect of daily blood collection from the tail vein on the course of pregnancy of aged animals (Holinka, Flurkey and Finch, unpublished). Of the 22 inseminated mice, only 7 came to term, but had predominantly stillborn fetuses. Since this fertility level is markedly below that of non-stressed mice of comparable age (Holinka *et al.*, 1978), it was therefore not possible to correlate insemination with subsequent success of pregnancy in this group.

[e]Pregnancy success was determined by the presence of implantation sites at laparoscopy. Implantation sites can be reliably identified only at day 6 of gestation and thereafter (Holinka *et al.*, 1979a). These numbers include therefore only animals on or after day 6 of gestation. The day of the plug was counted as Day 1.

retired breeders in experimental groups 6 and 8 (Table 1) were obtained at 8 mos of age after previous continuous breeding at the Jackson Laboratory. Mice were kept in a limited access colony designed for long-term aging studies in order to minimize the influence of infectious disease on age-related changes and help standardize breeding performance. Reproductive functions of female rodents are very sensitive to environmental factors such as light, temperature, population density, presence of males of the same strain or rodents of different strains (Rafferty, 1970). Mice were quarantined for at least one week and sampled for health screening, including gross and histopatholgic examination. Mice were then housed 5/cage in 12 × 13 × 30 cm plastic cages until mating. Room temperature was 24 ± 2 C; a 12-hour light cycle began at 0800; Purina Lab Chow® and water were available ad libitum. Two females were mated with one male (3–8 mo old) and checked daily for evidence of insemination by the presence of coital plugs before noon. Inseminated mice were housed singly and given a breeding pouch for nest building. Successful pregnancy was indicated at laparoscopy by the presence of implantation sites on day 6 and thereafter (day 1 is the day when the coital plug was first found), or by subsequent birth.

RESULTS AND DISCUSSION

These data are based on long-term observations of 1,292 female C57BL/6J mice from 9 cohorts of different birth dates which were used in studies of reproductive aging (Holinka and Finch, 1978; Holinka *et al.*, 1978, 1979a, 1979b). Mice are identified as experimental Groups 1–8 (Table 1), which in some cases include the same cohort at different ages and reproductive experience as indicated. The results describe three aspects of pregnancy in relation to age and reproductive history: (1) effects of virgin status on the mating interval required for insemination; (2) effects of age on mating resulting in pregnancy; and (3) incidence of pregnancy after insemination.

Vol. 16 1981

Effects of virgin status on the interval between first exposure to the male and insemination

Virgin mice, aged 3–12 mo, required longer periods of exposure to males for insemination than did previously parous mice (Table 1). For example, in Group 3 (8 mo old; first pregnancy up to 4 mo earlier), 90% were inseminated within 6 days, whereas only 33% of virgins, Group 1 (3.5 mo old) were inseminated after 14 days of continuous exposure to a male ($p < .01$, X^2 test with 1 df). Since estrous cycles are typically 4–6 days long at ages 3–8 mo in this colony (Nelson *et al.*, 1981), most previously parous mice were inseminated within 1 estrous cycle. A similar trend for effects of parity is seen in mice aged 12 mo, in which only 59% of virgins (Group 5) were inseminated, whereas 83% of previously parous mice (Group 4, same cohort and age) were inseminated in 18 days of exposure to a male ($p < .05$). It is possible that seasonal effects are also involved, but this issue can not be decided from the present data.

Given a sufficiently long exposure to a male, most young virgin mice can be inseminated: In Group 2a (3–5 mo) 93% were inseminated during 7 weeks exposure; this frequency is equivalent to that of other young mice at second pregnancy (Group 7). It is not known if the refractory subgroup of virgin mice (ca. 10%) ultimately can become inseminated (it is pertinent that the male mice mated with this group were established breeders). The reason why virgin mice require a longer mating period for insemination is unknown. Previous parity does increase the incidence of regular 4-day estrous cycles in young mice (preliminary observations, K. Flurkey and C.E. Finch, unpublished observations). Since most young virgin mice would be expected to have at least one proestrus during the 13 day mating period in which only 33% of Group 1 were inseminated, other unknown factors may be involved. The congenital vaginal diverticulum found in 4% of C57BL/6J mice (Cunliffe-Beamer and Feldman, 1976) might influence effectiveness of mating or of

seminal plug retention; however, mice with vaginal diverticula were excluded from these experiments.

Absence of age effects on the incidence of insemination

Age did not alter the fraction of matings in previously parous mice leading to insemination, which was > 80% [Groups 6 and 8 (11–12 mo old) and Group 7 (3–7 mo old)]. Additionally cohort 1015 performed similarly at different ages: the second pregnancy at 8 mo (Group 3) and third at 12 mo (Group 4) occured in 90% and 83% of mice, respectively. In Group 4 pregnancy was determined by the presence of implantation sites. Since fewer 12 mo old virgins (Group 5) of this same cohort were inseminated (59%, $p < .05$) during 18 days of exposure to males, the effects of age on the ability to become pregnant within 2 estrous cycles appear to be smaller than the effects of virgin status.

The failure of pregnancy after insemination

The failure of pregnancy after insemination varied from 9 to 31% as judged by the absence of implantation sites after day 6 or by the absence of parturition. No age trend emerged in the present groups (Table 1). This variable failure of pregnancy contrasts with the relatively unaltered mating efficiency leading to insemination in previously parous mice up to 12 mo of age (see above). Major age effects on the fertility of 11–12 mo-old-mice include smaller litters at parturition due to increased resorptions in most litters (Holinka *et al.*, 1979a) and increased stillbirths (Holinka *et al.*, 1978). The fraction of inseminated mice with implantation sites was not altered by age, 3–12 mo (Holinka *et al.*, 1979a).

In general, more virgin mice failed to become pregnant after insemination: At 3–8 mo, virgins had a mean failure of 19% (Group 1, Group 2a, average), whereas previously parous mice had mean failure of about 11% (Group 2b, Group 3, Group 7 average). At 11–12 mo, virgins had a mean failure of 31% (Group 5), whereas previously parous mice had a mean failure of 20% (average of Group 4, 29% and Group 8, 11%). Possible factors in the larger failure rate in Group 4 vs Group 8 include duration of exposure to males or seasonal effects although in the related C57BL strain litter size was not influenced by season (Franks and Payne, 1970).

The failure of young nulliparous mice to become pregnant after insemination (Group 2a) does not necessarily indicate permanent infertility. All previously inseminated, but nonpregnant mice (20% of Group 2a, prior virgins) became pregnant on second mating (Group 2b). It is possible that mating or pseudopregnancy in the inseminated-nonpregnant mice improves their reproductive performance during second mating. The data also suggest that the failure rate of pregnancy is similar in virgins inseminated only after prolonged, 7-week exposure to males (20%, Group 2a) and in the subgroup inseminated within 2 weeks (17%, Group 1). This result indicates that reproductive studies with virgin mice are not biased seriously by omitting the subgroup which fails to be rapidly inseminated after exposure to a male.

The reason for pregnancy failure in about 10% of previously parous, inseminated mice remains obscure. Plasma progesterone on days 6–10 after insemination in mice lacking implantation sites was about 50% lower than in mice with implantation sites (Holinka *et al.*, 1979a). These low progesterone levels are clearly distinguished from the elevated progesterone of pseudopregnancy in rats (Pepe and Rothchild, 1974). It is therefore possible

that functional corpora lutea of pregnancy may not have formed after ovulation in some inseminated mice which failed to show implantation sites. Alternatively, mating may have occured during an anovulatory cycle; anovulatory cycles are rare in young mice and increase to about 20% in mice aged 13–14 mo, as detected by the absence of tubal ova at estrus in singly housed (not mated) mice (R. Gosden, unpublished). A definite factor contributing to the failure of pregnancy after implantation in older mice is the presence of a subgroup (6%) in which the entire litter undergoes resorption after gestation day 12 (Holinka *et al.*, 1979a; Gosden *et al.*, 1980); complete resorption of all fetuses is extremely rare in young, previously parous mice and could not account for more than 1% of their failure to become pregnant.

SUMMARY

These data indicate that the efficiency of mating (based on the incidence of insemination) does not change significantly between the ages of 3 and 12 mo, regardless of previous reproductive history, provided that virgins are exposed to males for an extended period of time. Although virgins require a significantly longer mating period to achieve their first insemination than do previously parous mice, the failure rate of pregnancy is not influenced by the lag between exposure to the male and insemination. The failure to initiate pregnancy (implantation) after insemination varies considerably, tends to be higher in virgins, and may be unaffected by age up to 12 mo. Virgin mice which were inseminated and failed to become pregnant were shown to be fertile on second mating. These results suggest that studies of reproductive aging will not be seriously biased by ignoring the minor subgroup which fails to become rapidly inseminated after mating.

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Vol. 16 1981

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INFLUENCE OF AGING ON THE SURFACE AREA OF THE SMALL INTESTINE IN THE RAT

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Vol.
16
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INTRODUCTION

INTESTINAL SURFACE area is an important determinant of the rate of absorption of ingested nutrients. Since the absorptive surface area of the intestine has been difficult to measure, experimental results are usually expressed as absorption per total body weight (Deren, 1968) per unit of intestinal weight or per unit of intestinal length. However, it has not been established whether any of these parameters actually reflect the intestinal surface area. The purpose of this study was to develop a simple method of measuring the surface area of the intestine and study the influence of aging on the surface area of the small intestine.

MATERIALS AND METHODS

Animals

Males Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass.) were raised in our aging animal colony. They were allowed free access to chow and water. The rats varied in age from 2.5 to 92 weeks and were randomly selected for the study. Three to 5 rats were studied in each group.

Methods

In order to assure complete intestinal emptying and avoid unnecessary manipulation of the bowel, the rats were fasted for 48 hours before experimentation. On the day of the study, each rat was weighed and anesthetized with ether. Its abdomen was opened, and ligatures were secured at the pylorus and ileocecal valve. An inflow cannula was inserted into the duodenum distal to the pylorus and the outflow cannula was inserted into the terminal ileum just proximal to the ileocecal ligature. In order to eliminate the artifactual stretching of the bowel, intracorporeal fixation (Hromaidkova and Skala, 1963) of the intestine was performed. Bouin's solution (750 ml of saturated picric acid 1.22%, 250 ml of full strength formalin and 50 ml of Glacial acetic acid) was continuously perfused through the inflow cannula and irrigated the whole length of the small intestine. Fifteen minutes later, the intestine was perfused twice with normal saline and cleared of the Bouin's solution. The organ was then dissected along the mesenteric attachment and separated from the stomach proximally and the cecum distally.

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The following parameters were assessed in each specimen:

1. *Volume* of the small intestine was determined after the bowel had been placed on a flat surface and the inflow cannula had been attached to a syringe pump which infused normal saline into the intestinal lumen. The outflow cannula was attached to a glass t-tube. Adopting the "connecting containers" concept, this system allowed us to control the filling pressure of the small bowel. The volume of administered solution was determined and recorded. The intestinal contents were then emptied into a graduated cylinder and the recovered volume was measured to an accuracy of ± 0.1 ml. The procedure was repeated at least twice on each specimen and the mean value of the two measurements was calculated. The administered and recovered volumes were nearly identical.
2. *Length* was measured 5 minutes after the intestine had been suspended from the proximal end and an 8 gram weight attached to the distal end in order to ensure a constant degree of stretching of the bowel.
3. *Anatomical surface area* of the intestine was calculated by the following equation:

$$S = 2\sqrt{\pi L V}$$

S is the intestinal surface area, π is the ratio of the circumference of a circle to its diameter, L is the length of the small intestine in centimeters and V is the volume of the small bowel in mililiters. This formula was derived from integration of the standard formulae for measuring the volume and surface of a cylinder:

$$\text{Surface} = 2\pi rL$$
$$\text{Volume} = \pi r^2 L$$

where (r) is the radius and (L) the length of the cylinder.

4. *Dried length and weight* of the intestine was assessed 72-hours later.

RESULTS

Table 1 lists the animal weight, length, dry weight, and surface area of the small intestine in 36 rats of various ages. The intestinal length varied from 79 to 137 cm. A linear relationship between the length of the intestine and the age of the rats was found only in the animals younger than 6-weeks ($Y = 0.72\ X - 41.23$, $r = 0.98$). During the following 86 weeks, no statistically significant changes occured in the intestinal wet length. A similar relationship was noted between age and the dry length of the small bowel, although the dry length was usually 10% shorter than the wet length.

The mean intestinal surface area varied from 82 to 171 cm². The intestinal surface area increased linearly during the first 6 weeks of life and reached a plateau afterward (Fig. 1).

TABLE 1. MEAN ± SE VALUES FOR INTESTINAL LENGTH, WEIGHT, SURFACE AREA AND ANIMAL WEIGHT

Age (weeks)	Animal weight (grams)	Intestinal length (cm)	Intestinal dry length (cm)	Intestinal dry weight (gm)	Surface area (cm²)
2½	61 ± 5	79 ± 2	73 ± 2	2.9 ± 0.1	82 ± 2
3	78 ± 4	87 ± 4	81 ± 4	4.2 ± 0.4	97 ± 7
4	64 ± 13	92 ± 3	82 ± 3	3.1 ± 0.6	108 ± 13
5	96 ± 8	109 ± 0	95 ± 3	3.2 ± 0.7	125 ± 4
6	177 ± 8	114 ± 4	104 ± 0.5	4.4 ± 0.1	142 ± 4
11	314 ± 0	100 ± 5	93 ± 4	3.4 ± 0.2	144 ± 3
13	430 ± 13	113 ± 6	107 ± 4	5.8 ± 1.0	142 ± 10
20	520 ± 14	124 ± 6	105 ± 4	4.0 ± 0.6	163 ± 5
38	616 ± 12	129 ± 5	118 ± 8	10.0 ± 1.2	155 ± 8
40	581 ± 3	126 ± 4	108 ± 1.5	6.9 ± 0.3	146 ± 5
49	613 ± 11	137 ± 3	123 ± 3	9.0 ± 0.5	163 ± 9
64	636 ± 10	134 ± 10	121 ± 10	8.0 ± 1.2	171 ± 14
92	654 ± 12	131 ± 4	120 ± 4	8.4 ± 1.0	156 ± 8

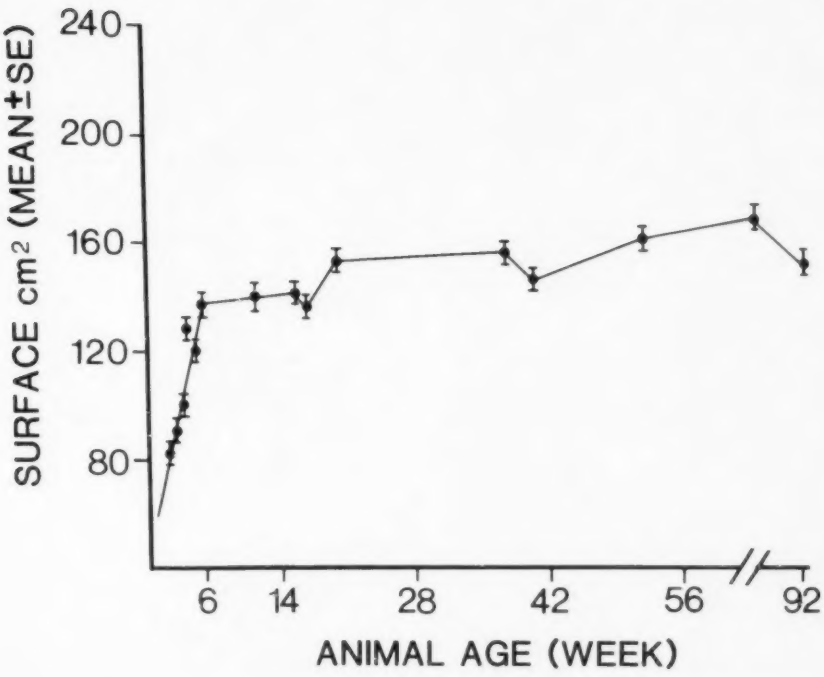

FIG. 1. The surface area of the small intestine in rats of all age groups.

Vol. 16 1981

No signficant increase (> 0.5) was noted in the intestinal surface area during the following 86-weeks of the life of the animals. There was no relationship between the small bowel surface area and the dry weight of the intestine (Fig. 2) or the animal's body weight. However, a strong linear correlation was found between the intestinal length and the surface area of the small intestine (Fig. 3) in rats of all age groups ($Y = 32.46 + 0.58X$, $r = 0.90$).

In order to assess the reproducibility of the volume measurement, in a separate study, we measured the intestine volume from 6 to 11 times in 3 rats of different age groups under similar conditions. The intestinal volumes of groups of animals of different ages (mean ± SD) were 16 ± 0.47, 16 ± 0.48 and 12 ± 0.37 ml respectively and indicated a standard deviation of less than 3%.

DISCUSSION

The surface area of the small intestine in rats of different ages was measured by determination of the volume required to fill the bowel under standardized conditions of stretch and pressure. In order to eliminate the problem of manipulation which leads to artifactual lengthening of the intestine and the reductions of its circumference, the bowel was fixed *in situ* in the anesthetized animal. We recognize that the small intestine is not a simple cylinder, but is composed of multiple villous projections and convolutions. However, there is ample evidence to support the validity of calculations that treat the intestinal surface area

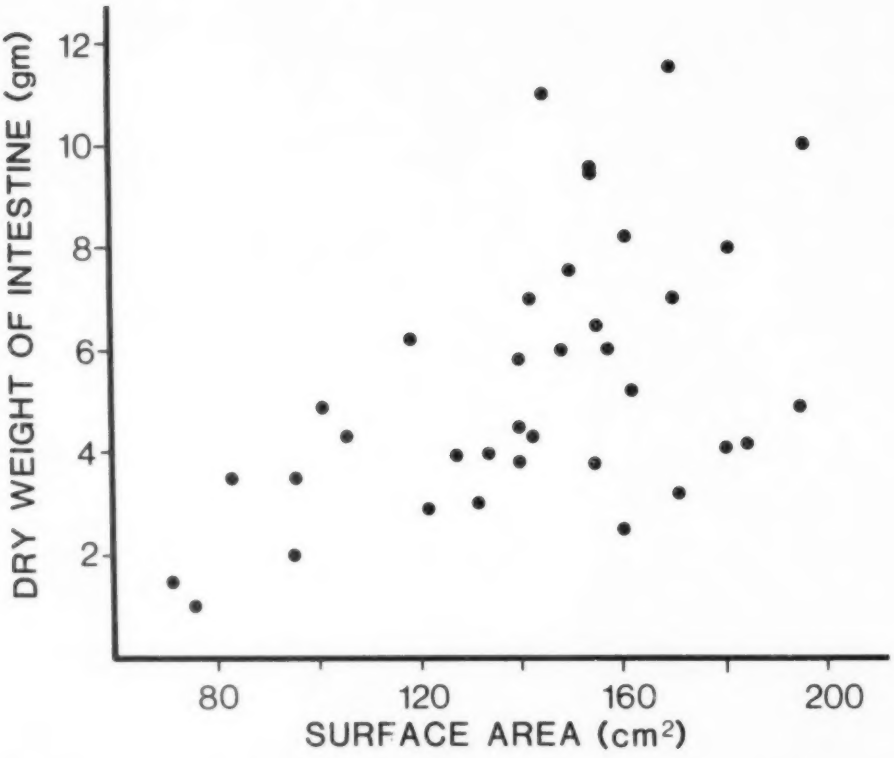

FIG. 2. The relationship between the small bowel surface area and the dry weight of the intestine.

as a simple cylinder. The work of Wilson and Dietschy (1974) and Westergaard and Dietschy (1974) clearly demonstrated that during absorption the central lacteals dilate and cause villi to swell laterally and obliterate the intervillous spaces. This swelling and effacement of intestinal interstices transforms the small intestinal mucosa into a flat surface. This transformation justifies treating the absorptive surface area of the small intestine as that of a simple cylinder. Work by Winne (1976) lends further support to this contention. The present method, which assesses the surface area, is technically easier than the previously described methods of morphometric measurements (Hromaidkova and Skala, 1963; Wood, 1944) and yields extremely reproducible results. Experimental data of intestinal absorption, which is frequently expressed as absorption per experimental animal or absorption per unit of body weight, could be more meaningfully expressed as transport per unit of surface area measured with the present methods.

The present data indicate that the surface area and the length of the small intestine increase up to 6 weeks of age which coincides with the age of maturation of the rat (Altman and Dittmar, 1962). As the rats grow older, both intestinal surface area and length fluctuate at their 6 week values and do not change with the increasing age of the rats. In other words, beyond 6 weeks of age, there is no significant change in the length and the surface area of the small intestine in spite of continuous increase in the animal's body weight.

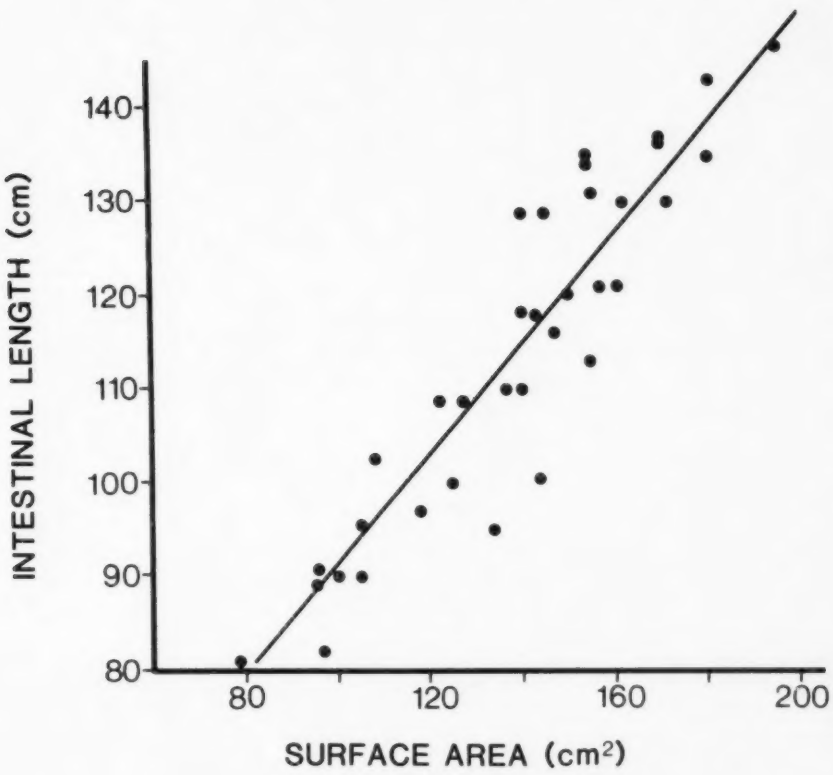

FIG. 3. The relationship between the length and the surface area of the small intestine.

Therefore, experimental data of intestinal absorption which is frequently expressed either as absorption per experimental animal or absorption per unit of body weight (Deren, 1968) are in error after 6 weeks of age in the animals. Our data indicates that both methods introduce errors since the intestinal surface area does not correlate with the body weight or intestinal weight. The strong linear correlation between the surface area and the length of the small bowel is explained only on assumption that either the average diameter of the small bowel in rats of different ages remains unchanged or, more likely, a linear correlation between the length and the diameter of the small bowel also exists.

In conclusion, the data herein reported indicates that assessment of the surface area of the intestine utilizing volume and length measurements is simple and can be used interchangeably with intestinal length to express the absorptive data in the rat. This parameter is preferred to indirect measurements like intestinal dry weight of body weight. Moreover, both the surface area and length fluctuate at their 6-week values and do not change with the increasing age of the rats.

SUMMARY

The conflicting reports regarding the influence of aging on intestinal absorption are due in part to the lack of a valid measurement of the intestinal surface area. The surface area

of the small intestine in male Sprague-Dawley rats (2.5 to 92 weeks of age) was measured by determination of the volume required to fill the bowel under standardized conditions of stretch and pressure, and by calculating the surface area using the volume and length of the bowel. The intestinal surface area of rats of differing ages varied from 82 to 171 cm^2. The intestinal surface area increased linearly with the age of the animals during the first 6 weeks of life. No further increase was noted in the intestinal surface area during the following 86 weeks of life. A strong linear correlation was found between the intestinal length and surface area in rats of all ages. In contrast, no relationship was found between the intestinal surface area and its dry weight or the animal's body weight. It is concluded that assessment of the intestinal surface area is simple and can be used interchangeably with intestinal length to express the absorptive data in the rat. This parameter is preferred to indirect measurements like intestinal dry weight or animal body weight. Moreover, both the surface area and the length fiuctuate at their 6-week values and do not change significantly with the increasing age of the rats.

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ACTIVITY OF GUANYLATE CYCLASE OF VARIOUS TISSUES AND ITS *IN VITRO* MODULATION BY EFFECTORS AS A FUNCTION OF AGE OF RATS

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Abstract—The activities of guanylate cyclase of the soluble, microsomal, mitochondrial and nuclear fractions of the cerebral cortex, heart and lungs of 4- (young), 34- (adult) and 85-week-old male rats were studied. The effects of aminophylline, histamine and estradiol on the enzyme were also determined.

The activity of the enzyme is high in the particulate fractions of the heart and lungs in all the ages, but in the cerebral cortex, the activity is high in the soluble fraction of young and old rats. Its activity is the highest in all the fractions of all the tissues of adult rats, except that of the soluble fraction of the cerebral cortex in which it is lowest at this age.

The particulate and soluble guanylate cyclase of the cerebral cortex, heart and lungs have different sensitivities to aminophylline, histamine and estradiol *in vitro*. In general, its sensitivity to the three effectors is the lowest in old age.

INTRODUCTION

GUANYLATE CYCLASE (GTP pyrophosphate lyase (cyclizing) EC 4.6.1.2) (GC) catalyses the formation of cyclic 3′-5′-guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). Cyclic GMP is involved as an alternate "second messenger" (Sutherland *et al.*, 1965). Numerous workers have studied the subcellular distribution of GC in various tissues (Hardman and Sutherland, 1969; Sulakhe *et al.*, 1976). Establishment of the precise subcellular distribution of GC may clarify the possible involvement of cGMP as an intracellular effector or "messenger" for biological signals. The levels of enzymes and their isoenzymes may govern various phases of the life span of an organism such as growth, reproduction and senescence. Variations in the activity of GC during insect development have been reported (Catalan *et al.*, 1976). There are relatively few reports correlating the activity of GC with the age of mammals.

Certain physiological agents such as calcium are known to play a major role in the regulation of GC activity in the cell (Goldberg *et al.*, 1976). Inhibition of cGMP-dependent phosphodiesterase increases cGMP level. Aminophylline is reported to increase the endogenous cGMP level by inhibiting phosphodiesterase (Appleman *et al.*, 1976). It is also known to increase the Ca^{++} level in the cell. Histamine increases the membrane

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permeability and allows the common inorganic ions (mainly cations) to flow down the electrochemical gradient (Ganellin, 1978; Douglas *et al.*, 1967). The action of estradiol is mediated through cytosol receptor whose level decreases with age (Kanungo *et al.*, 1975). A study of the effects of these effectors on GC may throw some light on the role of cGMP as a second messenger.

We describe here the subcellular distribution of GC in the brain, heart and lungs, and modulation of its activity by aminophylline, histamine and 17β-estradiol as a function of age of the rat.

MATERIALS AND METHODS

Animals

Immature (4−), adult (38−) and old (85-week) male rats of Wistar strain were used. They were maintained at 24 ± 2°C with artificial illumination for 12 h followed by a dark period of 12 h. They were fed with gram (*Cicer arietinum*) and freshly prepared diet containing flour and vitaminized powder milk in the ratio of 4:1 in water with added common salt. Tap water was given *ad libitum*.

Chemicals

All the chemicals used were of analytical grade and were obtained from Sigma Chemical Co., U.S.A. 8−^{14}C-GTP (sp. act 52 mCi/mmole) and 8−^{14}C-cGMP (sp. act 59 mCi/mmole) were obtained from Radiochemical Centre, Amersham, England. PPO (2,5-diphenyloxazole) and POPOP 1,4-bis (2,5-phenyloxazolyl) benzene were purchased from Koch-Light Laboratories Ltd., England.

Tissue preparation

The rats were killed by cervical dislocation at a fixed time (1600 h) of the day to avoid possible circadian rhythm of the enzyme. The brain, heart and lungs were taken out immediately and washed in cold normal saline to remove the adhering blood, and then blotted. The cerebral cortex and the ventricles of the heart were separated. The lungs were cleaned off cartilaginous tissue. The tissues were cut into slices of 0.2 - 0.4 mm thickness (Takagaki *et al.*, 1959).

In vitro study of the effects of effectors on the activity of GC was carried out by transferring 0.1 g of the sliced tissue into Warburg flasks containing 4.0 ml of incubation medium (De Robertis and Craven, 1976). The incubation medium was prepared by bubbling a mixture of 95% O_2 and 5% CO_2 into Krebs-Ringer bicarbonate buffer (pH 7.4) (Krebs and Henseleit, 1932). The flasks were then incubated in a water bath at 37°C for 15 min with shaking. The effectors (aminophylline, histamine and 17β-estradiol-final concentration 4×10^{-6} M) were added to the flasks and the incubation was continued for 60 min at 37°C with shaking. Control flasks were set up without effectors.

Subcellular fractionation

After 60 min of incubation, the slices were washed twice in fresh incubation medium to remove the effectors. A 10% homogenate of the slices was made in ice-cold 0.32 M sucrose (containing 5 mM Tris-HCl buffer, pH 7.4) in case of the cerebral cortex, and 0.25 M sucrose (5 mM Tris-HCl buffer, pH 7.4) for cardiac muscle and lungs. The homogenate was centrifuged at 1000 × *g* in an IEC PR-6 model refrigerated centrifuge. The supernatant was centrifuged at 17,000 × *g* for 55 min and the resulting pellet constituted the mitochondrial fraction. The supernanat was further centrifuged at 105,000 × *g* for 60 min in a VAC 601 ultracentrifuge. The pellet so obtained constituted the microsomal fraction, and the supernatant constituted the soluble fraction. The mitochondrial fractions were washed twice and suspended in 2- and 0.5 ml of the homogenizing medium, respectively, and used as the enzyme source.

Enzyme assay

Radiometric assay of GC of the three subcellular fractions of the cerebral cortex, heart and lungs was carried out by a slight modification of the method of Krishnan (1976). 0.1 ml of the reaction mixture contained 40.0 mM tris-HCl buffer (pH 7.4), 15 mM creatine phosphate, 40 μg creatine kinase, 3.3 mM $MnSO_4$, 10.0 mM

theophylline, 1.0 mM cGMP, 100-200 μg protein and 0.06 μmole ^{14}C-GTP (0.02 μCi/nmole). The reaction was carried out at 37°C in glass centrifuge tubes. The enzyme was preincubated at 37°C for 10 min. Then the reaction was started by adding ^{14}C-GTP. The reaction was continued for 10 min and then terminated by adding 20 μl of 30 mM EDTA (pH 7.6). Parallel blanks were run using water in place of enzyme. Parallel standards were also run with the experimental sets by using ^{14}C-cGMP, the product, to find out the column efficiency.

Isolation of cGMP

^{14}C-cGMP formed during the reaction was isolated by using neutral alumina column (White and Zenser, 1971). After stopping the reaction, the mixture was diluted to 0.5 ml with double distilled water, cooled and then centrifuged at 700 × *g* for 10 min. Glass columns, 15 cm in length and 9 mm inner diameter, were used. The column tip was packed with glass wool. Approximately 1.0 g of alumina was washed into each column with 15–20 ml of 0.1 M imidazole-HCl buffer (pH 7.0). The diluted supernatant was loaded on to the washed column. The cyclic nucleotides were eluted by 0.1 M tris-HCl buffer (pH 7.4) and 1.0 ml fractions were collected. Third, fourth and fifth fractions of the effluent contained cGMP which was pooled and counted for radioactivity with 10 ml of triton-toluene scintillator in an LS-100C Beckman liquid scintillation counter. Using ^{14}C-cGMP as standard, the column efficiency throughout was found to be around 60% which was used for correcting the recovery of ^{14}C-cGMP. The protein content was estimated (Lowry *et al.*, 1951), and the specific activity of the enzyme was calculated as picomoles of cGMP formed/min/mg protein.

Vol. 16 1981

RESULTS

GC activity of cerebral cortex, heart and lung

The total activity of guanylate cyclase is higher in the lung than in heart and cerebral cortex (Table 1). Among the four subcellular fractions, the microsomal and mitochondrial fractions show many-fold higher GC activity than the soluble and nuclear fractions except the soluble fraction of cerebral cortex of young and old rats. A marked increase in its activity occurs in all the fractions of heart and lungs up to adulthood, the increase being higher in lungs than in the heart. The activity decreases in all the fractions of heart and lungs after adulthood. In the cerebral cortex, the GC activity does not follow any particular pattern during aging. The soluble fraction of the young, the mitochondrial fraction of the adult and the nuclear fraction of the old show highest GC activity. There is an increase in GC activity in microsomal, mitochondrial and nuclear fractions of cerebral cortex up to adulthood which is followed by a decrease. In the soluble fraction the activity decreases till adulthood and then increases.

Effects of aminophylline, histamine and estradiol

Cerebral cortex. Aminophylline and estradiol stimulate the activity of GC of the particulate and soluble fractions of the brain of young rats. This effect markedly decreases or disappears with increasing age. Histamine inhibits the activity in all the fractions of young and old rats, whereas it stimulates that of the particulate fraction of the adult (Table 2).

Heart. Aminophylline stimulates the activity of GC of the particulate fraction of the heart of adult rats and inhibits that of young and old rats. It stimulates the soluble enzyme of young rats. This effect decreases with increasing age. Histamine inhibits the particulate enzyme of young and old rats, but stimulates that of the adult. Histamine inhibits the soluble enzyme at all ages. 17 β-estradiol stimulates GC of all the fractions of the heart of young rats, and this effect decreases with increasing age. Aminophylline and estradiol stimulate nuclear GC of all ages (Table 3).

TABLE 1. SPECIFIC ACTIVITY OF GUANYLATE CYCLASE (pmoles of ^{14}C-cGMP formed/min/mg protein) OF CEREBRAL CORTEX, HEART AND LUNG OF MALE RATS OF DIFFERENT AGES

Tissue	Age (weeks)	Microsomal		Mitochondrial		Soluble		Nuclear	
		Mean S.D.	p	Mean S.D.	p	Mean S.D.	p	Mean S.D.	p
Cerebral cortex	4	1.95 ± 0.07	< 0.05	3.45 ± 0.31	< 0.001	7.06 ± 0.95	< 0.001	2.02 ± 0.16	< 0.001
	38	2.25 ± 0.16 (+15%)	< 0.001	8.80 ± 0.79 (+155%)	< 0.001	1.77 ± 0.24 (−75%)	< 0.001	7.34 ± 0.70 (+263%)	< 0.02
	85	1.07 ± 0.11 (−52%)		3.02 ± 0.27 (−66%)		4.21 ± 0.35 (+138%)		5.58 ± 0.47 (−24%)	
Heart	4	2.01 ± 0.19	< 0.001	2.97 ± 0.18	< 0.01	0.81 ± 0.17	< 0.001	0.58 ± 0.03	< 0.01
	38	12.71 ± 1.43 (+532%)	< 0.001	4.72 ± 0.40 (+59%)	< 0.001	2.42 ± 0.26 (+199%)	< 0.001	0.75 ± 0.05 (+29%)	< 0.001
	85	2.01 ± 0.31 (−84%)		1.43 ± 0.20 (−70%)		0.23 ± 0.03 (−91%)		0.18 ± 0.01 (−76%)	
Lung	4	7.60 ± 0.90	< 0.001	7.02 ± 0.89	< 0.01	1.33 ± 0.12	< 0.001	0.89 ± 0.10	< 0.001
	38	53.46 ± 4.99 (+603%)	< 0.001	20.23 ± 3.79 (+188%)	< 0.01	3.73 ± 0.42 (+181%)	< 0.01	4.13 ± 0.44 (+364%)	< 0.001
	85	9.14 ± 1.00 (−83%)		1.78 ± 0.21 (−91%)		1.59 ± 0.17 (−57%)		0.50 ± 0.05 (−88%)	

Determinations were made as described in the text. Results are given as mean values from 4–6 rats. *S.D.*, standard deviation; p, significance of change.

Vol. 16 1981

TABLE 2. EFFECTS OF HISTAMINE, AMINOPHYLLINE AND ESTRADIOL ON SPECIFIC ACTIVITY OF GUANYLATE CYCLASE (pmoles of ^{14}C-cGMP formed/min/mg protein) OF VARIOUS SUBCELLULAR FRACTIONS OF CEREBRAL CORTEX OF MALE RATS OF DIFFERENT AGES

Fraction	Experimental condition	4 weeks		38 weeks		85 weeks	
		Mean S.D.	*p*	Mean S.D.	*p*	Mean S.D.	*p*
Microsomal	Slice	1.95 ± 0.07		2.25 ± 0.16		1.07 ± 0.11	
	Slice + His.	1.50 ± 0.19	< 0.02	4.02 ± 0.75	< 0.01	0.96 ± 0.24	NS
	Slice + Am.	4.71 ± 0.34	< 0.001	2.88 ± 0.17	< 0.01	1.48 ± 0.41	NS
	Slice + Est.	5.13 ± 0.53	< 0.001	2.87 ± 0.42	< 0.05	1.23 ± 0.39	NS
Mitochondrial	Slice	3.45 ± 0.31		8.80 ± 0.79		3.02 ± 0.27	
	Slice + His.	2.02 ± 0.21	< 0.01	12.51 ± 1.22	< 0.05	1.91 ± 0.21	< 0.01
	Slice + Am.	3.99 ± 0.24	< 0.05	9.38 ± 0.60	NS	3.73 ± 0.34	< 0.05
	Slice + Est.	6.78 ± 0.23	< 0.001	9.48 ± 0.51	NS	4.61 ± 0.51	< 0.01
Soluble	Slice	7.06 ± 0.95		1.77 ± 0.24		4.21 ± 0.35	
	Slice + His.	1.21 ± 0.02	< 0.001	1.49 ± 0.07	NS	0.34 ± 0.10	< 0.001
	Slice + Am.	15.81 ± 1.74	< 0.001	2.43 ± 0.32	< 0.05	0.45 ± 0.08	< 0.001
	Slice + Est.	9.87 ± 0.55	< 0.01	1.03 ± 0.09	< 0.01	0.10 ± 0.01	< 0.001

Concentrations of aminophylline (Am), histamine (His) and estradiol (Est) were 4×10^{-6}M in each case.

Determinations were made as described in the text. Results are given as mean values from 4–6 rats. *S.D.*, standard deviation, *p*, significance of change; NS, not significant.

Lung. Aminophylline, histamine and estradiol stimulate the activity of GC of the particulate fraction of adult rats. In the young and the old, either these effectors inhibit it or have no effect. Though none of the effectors have any significant effect on soluble GC of young and old rats, all of them inhibit it in the adult. The three effectors stimulate nuclear enzyme of young rats (Table 4).

DISCUSSION

The distribution of GC between soluble and particulate fractions is different in different tissues. Our data show that the activity of GC is high in particulate fractions, that is, the microsomal and mitochondrial, of the heart and lungs (Table 1). This indicates that in these tissues, the enzyme is predominantly membrane bound (Sulakhe *et al.*, 1976; White, 1975). Relatively high activity in the soluble fraction of the cerebral cortex shows that in the brain the enzyme is cytosolic (Goridis and Morgan, 1972).

The activities of GC of various subcellular compartments show a similar pattern of change as a function of age, except in the soluble fraction of the cerebral cortex. The enzyme activity is high in all the fractions of the heart and lungs of the adult. Cyclic GMP stimulates type a_2 protein kinase (Kuo and Greengard, 1970). Therefore, variations in the cGMP level brought about by changes in GC activity may affect protein kinase activity which may then alter phosphorylation of proteins including chromosomal proteins. This may alter the template activity of DNA. The decrease in GC activity in old age is consistent with the earlier finding (Williams and Thompson, 1973).

Aminophylline causes a rapid increase in the level of cyclic nucleotides (cAMP and cGMP) by inhibiting intracellular phosphodiesterases (Appleman *et al.*, 1973). It also raises the Ca^{++} level in the cell (Sulakhe *et al.*, 1976) which may be another reason for the increase in cGMP. Our data show that microsomal enzyme of the cerebral cortex of young rats is more sensitive to aminophylline than that of the heart and lungs. The mitochondrial GC of the lungs and heart of adult rat is more sensitive than that of the brain. The soluble enzyme of all the three tissues of young rats is stimulated by aminophylline. This effect decreases with increasing age. This shows differential sensitivity of particulate and soluble enzyme of the three tissues towards aminophylline at different ages. The differential effects of aminophylline on the particulate and soluble enzymes of the heart and lungs with increasing age may be due to the presence of different isoenzymes of GC in these tissues. It has been reported that soluble and particulate fractions of the homogenates of most rat tissues have two different forms of GC which differ in properties and molecular sizes (Kimura and Hurad, 1974; Chrisman *et al.*, 1975).

Histamine has different effects on the activity of GC of different tissues and of different age. Histamine is known to increase the membrane permeability and allows the common inorganic ions to flow down electrochemical gradients (Douglas *et al.*, 1967). Therefore, the stimulation of GC by histamine may be due to an increase in Ca^{++} level (Sulakhe *et al.*, 1976; Nakazawa and Sano, 1974) which flows into the cell from the incubation buffer due to an increase in membrane permeability. The activity of histamine is known to be mediated through either H_1 or H_2 receptors whose actions are different, if not opposite (Ganellin, 1978). Our results show that histamine inhibits GC of all the fractions of the three tissues, both in young and old rats. However, it stimulates GC of the particulate fraction of the adult. This may be due to changes in the relative concentrations

Vol. 16 1981

TABLE 3. EFFECTS OF HISTAMINE, AMINOPHYLLINE AND ESTRADIOL ON SPECIFIC ACTIVITY OF GUANYLATE CYCLASE (pmoles of ^{14}C-cGMP formed/min/mg protein) OF VARIOUS SUBCELLULAR FRACTIONS OF HEART OF MALE RATS OF DIFFERENT AGES

Fraction	Experimental condition	4 weeks		38 weeks		85 weeks	
		Mean S.D.	p	Mean S.D.	p	Mean S.D.	p
Microsomal	Slice	2.01 ± 0.19		12.71 ± 1.43		2.01 ± 0.31	
	Slice + His.	1.48 ± 0.12	< 0.02	14.68 ± 1.66	NS	1.35 ± 0.16	< 0.05
	Slice + Am.	1.63 ± 0.12	< 0.05	16.54 ± 1.98	NS	1.93 ± 0.23	NS
	Slice + Est.	3.03 ± 0.22	< 0.01	15.95 + 1.11	< 0.05	0.41 ± 0.05	< 0.001
Mitochondrial	Slice	2.97 ± 0.18		4.72 ± 0.40		1.43 ± 0.20	
	Slice + His.	2.06 ± 0.17	< 0.01	8.79 ± 0.91	< 0.01	0.73 ± 0.09	< 0.01
	Slice + Am.	1.67 ± 0.04	< 0.001	10.11 ± 1.22	< 0.01	0.40 ± 0.05	< 0.001
	Slice + Est.	5.35 ± 0.46	< 0.05	7.19 ± 0.82	< 0.01	1.39 ± 0.17	NS
Soluble	Slice	0.81 ± 0.17		2.42 ± 0.26		0.23 ± 0.03	
	Slice + His.	0.08 ± 0.03	< 0.01	1.93 ± 0.20	NS	0.06 ± 0.01	< 0.001
	Slice + Am.	1.51 ± 0.16	< 0.01	1.89 ± 0.21	NS	0.02 ± 0.02	< 0.001
	Slice + Est.	4.31 ± 0.52	< 0.001	2.54 ± 0.21	NS	0.17 ± 0.02	NS

Concentrations of aminophylline (Am), histamine (His) and estradiol (Est) were 4×10^{-6}M in each case.

Determinations were made as described in the text. Results are given as mean values from 4–6 rats. *S.D.*, standard deviation, *p* significance of change; NS, not significant.

TABLE 4. EFFECTS OF HISTAMINE, AMINOPHYLLINE AND ESTRADIOL ON SPECIFIC ACTIVITY OF GUANYLATE CYCLASE (pmoles of ^{14}C-cGMP formed/min/mg protein) OF VARIOUS SUBCELLULAR FRACTIONS OF LUNG OF RATS OF DIFFERENT AGES

Fraction	Experimental condition	4 weeks		38 weeks		85 weeks	
		Mean S.D.	*p*	Mean S.D.	*p*	Mean S.D.	*p*
Microsomal	Slice	7.60 ± 0.90		53.46 ± 4.99		9.14 ± 1.10	
	Slice + His.	7.15 ± 0.89	NS	84.75 ± 9.92	< 0.01	9.12 ± 1.78	NS
	Slice + Am.	6.49 ± 0.71	NS	82.16 ± 7.66	< 0.01	9.02 ± 1.12	NS
	Slice + Est.	4.41 ± 0.41	< 0.01	68.41 ± 7.21	< 0.05	8.56 ± 0.92	NS
Mitochondrial	Slice	7.02 ± 0.89		20.23 ± 3.79		1.78 ± 0.21	
	Slice + His.	3.74 ± 0.42	< 0.01	54.47 ± 6.21	< 0.01	1.94 ± 0.20	NS
	Slice + Am.	6.35 ± 0.67	NS	54.37 ± 6.30	< 0.01	1.86 ± 0.20	NS
	Slice + Est.	4.09 ± 0.41	< 0.01	44.92 ± 5.11	< 0.01	1.53 ± 0.10	NS
Soluble	Slice	1.33 ± 0.12		3.73 ± 0.42		1.59 ± 0.17	
	Slice + His.	1.56 ± 0.11	NS	1.38 ± 0.11	< 0.001	1.19 ± 0.12	< 0.05
	Slice + Am.	1.51 ± 0.15	NS	1.07 ± 0.10	< 0.001	1.29 ± 0.14	NS
	Slice + Est.	1.59 ± 0.17	NS	1.68 ± 0.18	< 0.01	1.29 ± 0.11	NS

Concentrations of aminophylline (Am), histamine (His) and estradiol (Est) were 4×10^{-6}M in each case.

Determinations were made as described in the text. Results are given as mean values from 4–6 rats. *S.D.*, standard deviation; *p*, significance of change; NS, not significant.

of H_1 and H_2 histamine receptors. Similar qualitative differences in the effect of histamine on ornithine decarboxylase of different tissues of the rat have been observed (to be published). Furthermore, the particulate and soluble GC of the three tissues seem to be differentially seensitive towards histamine, especially in adult rats.

Sex hormones are known to promote growth during development. Our data show that estradiol stimulates the activity of GC of all the fractions of the three tissues of young rats. This stimulatory effect is not seen in old rats. The only exception is seen in the particulate enzyme of the lung of the adult. The decrease in the stimulatory effect of estradiol with increasing age may be due to a decrease in the level of its cytosol receptor with increasing age (Kanungo *et al.*, 1975). Differential effects of estradiol on the particulate and soluble GC of the lungs indicate the existence of different isoenzymes of GC in this tissue (Kimura and Hurad, 1974; Chrisman *et al.*, 1975). Recently, it has been shown that androgens stimulate the activity of GC (Vesely, 1979).

The above studies show that (i) the activities of GC vary widely with the type of tissue, (ii) its activity is high in the brain, heart and lungs in the reproductive phase of the rat, (iii) the particulate and soluble GC of the three tissues appear to be different as indicated by the differences in their sensitivities to aminophylline, histamine and estradiol, and (iv) in general, there is a decrease in the sensitivity of GC to these three effectors in old age. Such age-dependent variations in the activity of GC may alter the level of cGMP which serves as a second messenger. Alterations in the levels of such effectors may change gene expression during development, growth and senescence as envisaged in the model for aging (Kanungo, 1975, 1980).

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Vol. 16 1981

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GENETICS OF LONGEVITY IN *DROSOPHILA*

V. The Specific and Hybridised Effects of *Rolled, Sepia, Ebony* and *Eyeless* Autosomal Mutants

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Vol.
16
1981

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INTRODUCTION

THERE IS SOME evidence that the life-span of *Drosophila* is under genetic control (Gonzales, 1923; Clarke and Maynard Smith, 1955; Parsons, 1966; Lints and Lints, 1968; Clark and Gould, 1970; Woodhams and Hollingsworth, 1971; Lints and Hoste, 1974; Biscardi and Webster, 1977; Gould and Clark, 1977; Parsons, 1978; Bozcuk, 1978; Lints, 1978; Lints et al, 1979; Ünlü and Bozcuk, 1979a, 1979b, 1979c; Bozcuk *et al.*, 1979 and Lints, 1980). The manner in which this control operates is not clearly understood, and only a limited amount of work has been done on the relationship between genetics and ageing (reviewed in Comfort, 1978 and Lints, 1978). Recently, Lints (1980) emphasized the importance of the search for mutants affecting some components of growth and the study of the way these mutants age and die. Furthermoe, Ünlü and Bozcuk (1979b) have stated that future studies of the control of life-span in mutants might well provide a deeper understanding of the genetic determination of longevity in *Drosophila*. For these reasons, it is thought that further analysis of the role of genes in the determination of adult life span is necessary.

This is, therefore, another in our series of investigations aimed at clarifying the effects of various mutants and genotypes upon the duration of life of adult *Drosophila*.

In this work, the specific effects of 4 types of autosomal mutants located on the 2nd (rolled), 3rd (sepia and ebony) and 4th (eyeless) pairs of chromosomes will be investigated in both sexes in relation to longevity. In addition to the pure lines, the reciprocal hybrids in the F_1 and the various reciprocal double-hybrids in the F_2 generations will also be examined in possible heterozygotic combinations. The aim is to see if the deleterious life-shortening effects of these inbred mutant genes can be counteracted by hybridisation.

This paper is dedicated to M. Kemal Atatürk, founder of Modern Turkey, on the centenary of his birth.

METHODS AND PROCEDURE

Flies

Four different autosomal mutant strains of *Drosophila melanogaster* were used in the experiments, Lindsey and Grell (1967) described these as follows:

Rolled (rl): Located on the 55.1[st] position of the 2nd chromosome-abbreviated as 2-55.1; phenotype: wing edges rolled downward, margins somewhat frayed, eyes are small, dark and rough.

Sepia (se): Location: 3-26.0; phenotype: Eye colour is brown at eclosion, darkening to sepia and becoming black with age. Pigmentation of ocelli is normal. The se/+ has more isoxanthopterins than +/+.

Ebony (e): Location: 3-77.7; phenotype: Body colour is shining black. Viability lowered to about 80% of wild type. May interact with other body colours.

Eyeless (ey^2): Location: 4-2.0; phenotype: Eye reduced to one half to one-fourth wild type-area, sometimes completely lost. Cephalic complex is smaller than normal.

A local stock of Hacettepe w.t. is used as control. This has relatively shorter mean life-span than Oregon w.t. (Bozcuk, 1978).

Crosses

In the first experiment life tables were constructed for the mutants *rolled*, *sepia*, *ebony* and *eyeless* together with Hacettepe w.t.. In the second experiment, which was almost contemporary to the first, reciprocal crosses were made between *e* ♀ × ey^2 ♂, ey^2 ♀ × *e* ♂, *rl* ♀ × *se* ♂ and *se* ♀ × *rl* ♂, F_1 generations of these were named CD, DC, AB and BA respectively. The phenotypes of the F_1 appeared to be normal and no selection was made during collection of experimental flies.

For the third experiment, four different groups of the F_1 hybrids were used in the reciprocal crosses. The offspring of the cross between CD ♀ and AB ♂ was named CDAB; between AB ♀ × CD ♂, ABCD; between DC ♀ × BA ♂, DCBA and between BA ♀ × DC ♂, BADC. Again all of the F_2 flies appeared to be normal and no selection was made during the collection of experimental F_2 flies.

Life Tables

The flies were kept constantly at 25°C on standard medium composed of maize-flour, agar, sucrose, dried yeast and a mold inhibitor. The humidity of the experimental cabinets was 40–60%. The flies were always in darkness, except during transfers on to fresh medium (twice weekly), when they were in light. Five flies were initially placed in 2.5 × 7.5 cm glass vials containing the food. All the flies were raised from parents whose ages were 5–10 days old and the females used in the experiments were virgins. The experimental populations could be considered as contemporaneous.

RESULTS

Initially life tables were constructed for both sexes of the four autosomal mutants as well as of w.t. Hacettepe as control. The results are summarised in Table 1.

It is seen in the table that the mean life spans of Hacettepe w.t. (62.72 and 56.64 days for males and females respectively) are quite similar to our earlier data (Bozcuk, 1978). The differences between the sex-mixed means of *rolled* and *sepia*, *ebony* and *sepia*, *eyeless* and *sepia* are all significant in favour of *sepia*, there is also a significant difference between control and *eyeless*, *ebony* and *rolled*. In fact, the sex-combined mean life-spans of the mutants *rolled* (43.96 days), *ebony* (46.29) and *eyeless* (46.69) flies were significantly less than that of the w.t. whereas *sepia* has a mean (58.82) similar to w.t.

There is a significant difference between the opposite sexes of *eyeless*, but this is not found in the other three mutants. The differences between the mean life span of *se* and

Vol. 16 1981

TABLE 1. MEAN LIFE SPANS OF THE AUTOSOMAL *Rolled, Sepia, Ebony* AND HACETTEPE W.T. OF *D. Melanogaster*

Class no.	Group name	Genotype and sex	No. of flies	Mean Life-span (days) ± *S.E.*	*S.D.*	Sex mixed life-span	Significant differences between some classes ($p < 0.05$)	
1	A	rolled (rl) ♂♂	96	44.28 ± 1.22	12.01	43.96	1–3	1–9
2		♀♀	52	43.65 ± 1.71	12.36		2–4	2–10
3	B	sepia (se) ♂♂	70	61.72 ± 2.13	17.82	58.82	3–5	3–7
4		♀♀	33	55.93 ± 3.13	17.97		4–6	4–8
5	C	ebony (e) ♂♂	89	45.88 ± 1.55	14.92	46.29	5–9	
6		♀♀	88	46.70 ± 1.39	13.08		6–10	
7	D	eyeless (ey^2) ♂♂	84	51.14 ± 2.02	18.55	46.69 (48.94)	7–8	7–9
8		♀♀	82	42.24 ± 1.65	14.98		8–10	
9	K	Hacettepe w.t (control) ♂♂	85	62.72 ± 1.41	13.06	59.68		
10		♀♀	75	56.64 ± 2.06	17.29			

S.E. Standard error of the mean, *S.D.*: Standard deviation.

those of *rl*, *e* and *ey*² males are all significant. Again, the mean life spans of *rl*, *e* and *ey*² females are significantly shorter than the *se* females. The mean for the w.t. males is significantly greater than those for the *rl*, *e* and *ey*² males; and the w.t. females are longer-lived than *rl*, *e* and *ey*² females.

The overall average of the life-spans of the 4 types of mutant (48.94 days) is 17.99% shorter than the sex-mixed average of the w.t. (56.68).

In order to illustrate the effects of these experimental genotypes on longevity, the survival curves are drawn and presented in Fig. 1. The forms of the curves of males and females in the *rl* and *e* are quite alike, whereas in the *se* and *ey*² they were somewhat different. The survival curves and the means for the sexes of the *ey*² mutant are very similar to those given earlier by Ünlü and Bozcuk (1979c).

Life table data for the second experiment is summarised in Table 2. As will be seen in the table the sex-combined means for the cross-type CD (*e* ♀ × *ey*² ♂) and AB (*rl* ♀ × *se* ♂) are quite similar (about 60 days) whereas they are 74.13 days for DC (*ey* ♀ × *e* ♂) and 69.75 days for BA (*se* ♀ × *rl* ♂). In other words, reciprocals of the CD and AB types of crossing produced hybrids differing very much in longevity. The differences between the sex-mixed means of CD and DC, CD and BA, AB and DC, AB and BA, are all significant at various levels. It is also noticed that the sex-mixed mean duration of life of the control group is shorter than those of the DC (ey²/ + e/ +) and BA (se/ + rl/ +) F_1 genotypes. Although there is a significant difference between the means of the sexes of BA hybrids, this is not so for other types of hybrids. However, there are significant differences between the males of the DC and CD, DC and AB, DC and BA groups, and between the females of the CD and BA, DC and AB, AB and BA groups. Three types of F_1 males (CD, AB and BA) are not longer-lived than the w.t. control males, but those of the DC group are longer-lived. However, F_1 females of the DC and BA groups are significantly longer lived than the w.t. females.

If we look at the survival curves for this life table data (Fig. 2) a clearer idea can be obtained. Both the characteristic and length of the curves have changed very much in comparison with the mutant parents. The hybridisation has caused more rectangular and extended curves. The longest lived individuals survived to about 90 days in the case of heterozygotes CD and AB, and to about 100 days in the case of heterozygotes DC and BA, whereas these figures were in the range of 69–83 days for the parent mutants.

It was further wondered whether the degree of vigour could be increased by additional hybridisation, and to see to what extent this would counteract the deleterious effect of the mutant genotypes. Therefore reciprocal hybridisation among the F_1 genotypes was undertaken, and as described in detail in the section on material and procedure, four types of double-hybrids were obtained: CDAB, ABCD, DCBA and BADC.

It is seen in Table 3 that the genotypes of the F_2 offspring are theoretically identical in each case. If we look at the sex-mixed means, they are 66.37 days for ABCD, 68.50 for CDAB, 70.19 for DCBA and 72.98 for BADC.

The differences between the sex-combined means of CDAB and BADC, ABCD and DCBA, ABCD and BADC, are significant. The sex-mixed means of all the F_2 progeny groups are significantly longer than that of the w.t. control. If we examine the sex differences between the means of each genotype in Table 3 it is seen that in three cases (ABCD, DCBA and BADC) females have a longer life span than the respective males, although in the CDAB group there is no significant difference. Only the males of the CDAB and BADC genotypes have significantly greater means than the control males

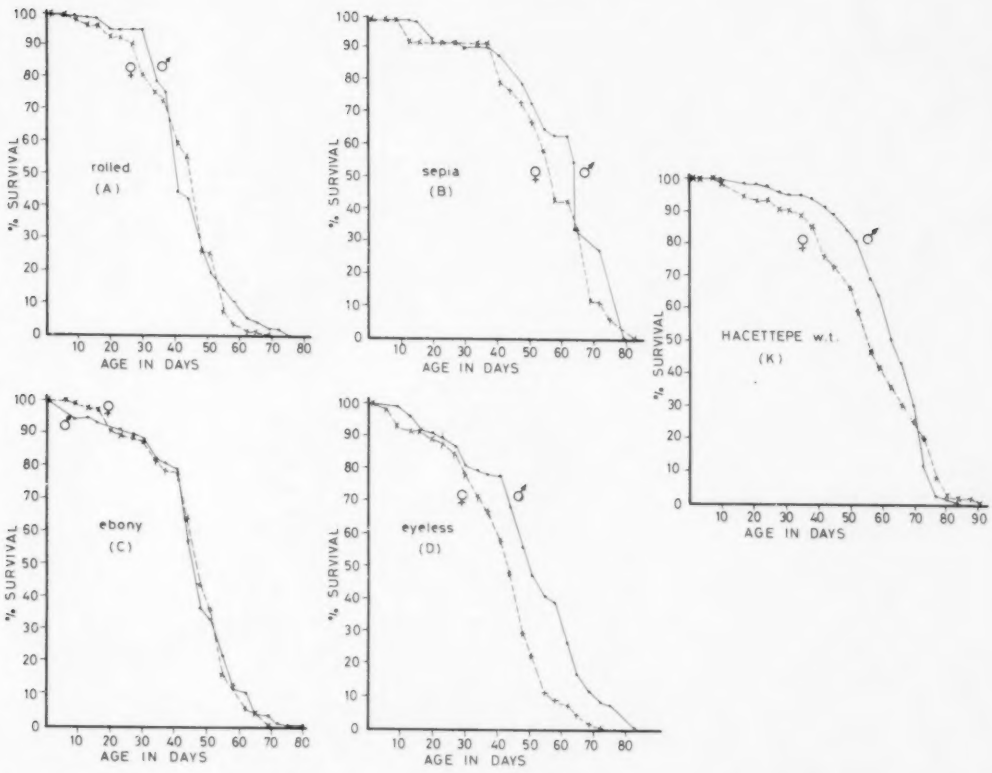

FIG. 1. Survival curves for the males and females of autosomal *rolled*, *sepia*, *ebony* and *eyeless* mutant strains and of Hacettepe w.t. *D. melanogaster*.

whereas females of the all F_2 genotypes show much extended life span against the control females at various levels of significance. The greatest mean belongs to BADC female (76.50 days) and the least to ABCD males (63.30 days) and w.t. females (56.64 days)

While the overall average life span of the 4 autosomal mutants was 48.94 days, it was lengthened to 66.32 days (35.5% increase) in the first generation hybrids, F_1, (CD, DC, AB and BA) and to 69.51 days (42.0% increase) in the second generation hybrids, F_2, (CDAB, ABCD, DCBA and BADC). The mean for the females of quadruple heterozygotes (72.13) increased by 27.3% against w.t. females (56.64), 53.1% against the mean of parental mutant females (47.1) and 70.7% against parental ey^2 females. Whereas for the mean of the males of quadruple heterozygotes (67.03) the increase is 6.8% against the mean of w.t. males (62.72), 32.0% against the average of parental mutant males (50.75) and 51.3% against *rl* males (44.28).

The survival curves for the populations studied in the third experiment are presented in Fig. 3. As can be seen in this and in previous F_1 figures, the curves are, in general, of quite rectangular type as described by Comfort (1978) and are demonstrative of typical senescence. However the survival curve of the *eyeless* mutant flies (in Fig. 1) in a way resembles the intermediate type (Rockstein and Miquel, 1973). Through two successive hybridisations more homogenous populations are gradually obtained. Actually, no deaths

TABLE 2. MEAN LIFE SPANS OF THE F_1 HYBRIDS OF FOUR DIFFERENT CROSSES OF *D. Melanogaster*

Class no.	Group name	Genotype of parents	F_1 genotype and sex	No. of flies	Mean life-span (days) ± *S.E.*	*S.D.*	Sex mixed life-span	Significant differences between some classes ($p < 0.05$)
1	CD	e/e ♀ X ey^2/ey^2 ♂	e/+ ey^2/+ ♂♂	74	58.24 ± 3.20	27.55	60.67	1-3 1-4 1-8
2		(C) (D)	e/+ ey^2/+ ♀♀	59	63.10 ± 2.32	17.86		2-3 2-8
3	DC	ey^2/ey^3 ♀ X e/e ♂	ey^2/+e/+ ♂♂	68	75.48 ± 1.92	15.89	74.13	3-7 3-9 3-10
4		(D) (C)	ey^2/+e/+ ♀♀	67	72.79 ± 2.13	17.43		4-10
5	BA	rl/rl ♀ X se/se ♂	rl/+se/+ ♂♂	67	60.79 ± 2.74	22.41	60.75	5-3 5-4 5-8
6		(A) (B)	rl/+se/+ ♀♀	70	60.71 ± 2.43	20.37		6-3 6-4 6-8
7	BA	se/se ♀ rl/rl ♂	se/+rl/+ ♂♂	67	63.95 ± 3.18	26.04	69.75 (66.32)	7-8
8		(B) (A)	se/+rl/+ ♀♀	68	75.55 ± 3.01	24.86		8-9 8-10
9	K	Hacettepe w.t	(Control) ♂♂	85	62.72 ± 1.41	13.06	59.68	
10			♀♀	75	56.64 ± 2.06	17.29		

Control (Hacettepe w.t.) is the same group as in Table 1.

were recorded until the 32nd day in the CDAB male and female flies of the F_2 hybrids (Fig. 3), and until the 25th day for the males and the 38th day for the BADC female hybrids. Maximum longevities recorded for 4 types of F_2 hybrids are very similar and around 90 days, which is somewhat shorter than those of F_1 hybrids, and longer than those of mutant parents. It is seen from the standard deviations in the three tables, and from the figures, that the most homogenous population is the F_2, and the most heterogenous population is the F_1.

DISCUSSION

In this work we have continued the study of the effects of autosomal mutant genes on

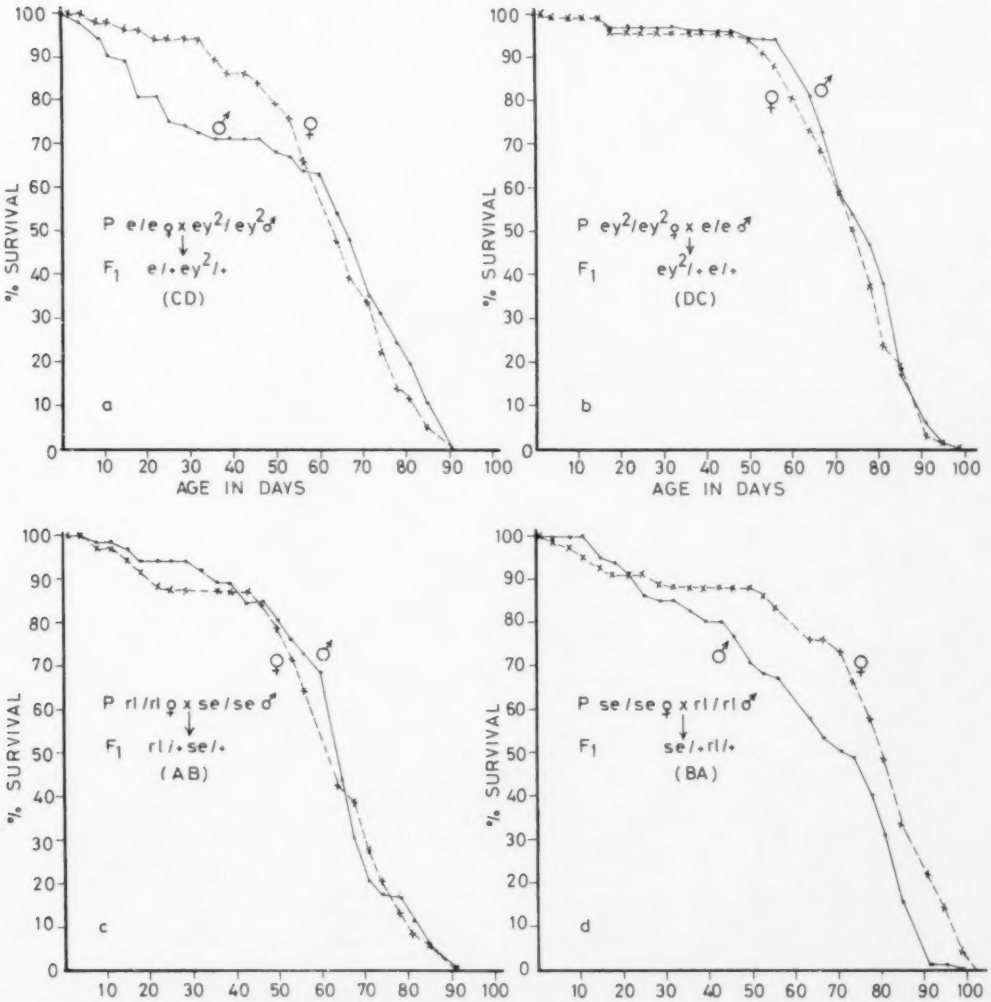

FIG. 2. Survival curves for the F_1 hybrid progenies produced by the crosses e ♀ X *ey²* ♂, *ey²* ♀ X *e* ♂, *rl* ♀ X *se* ♂ and *se* ♀ X *rl* ♂.

Vol. 16 1981

TABLE 3. MEAN LIFE SPANS OF THE F_2 HYBRIDS OBTAINED FROM THE CROSSES OF FOUR TYPES OF DOUBLE HYBRIDS

Class no.	Group name	Genotype of parents (F_1)	Genotype of F_2 offspring	No. of flies	Mean life-span (days) ± *S.E.*	*S.D.*	Sex mixed life-span	Significant differences between some classes ($p < 0.05$)		
1	CDAB	e/+ey²/+ ♀ X rl/+se/+ ♂	e/+ey²/+rl/+se/+ ♂♂	71	69.12 ± 1.49	12.55	68.50	1–8	1–10	
2		(CD) (AB)	e/+ey²/+rl/+se/+ ♀♀	78	67.88 ± 1.26	11.20		2–6	2–8	2–10
3	ABCD	rl/+se/+ ♀ X e/+ey²/+ ♂	rl/+se/+e/+ey²/+ ♂♂	76	63.30 ± 1.39	12.18	66.37	3–4	3–6	
4		(AB) (CD)	rl/+se/+e/+ey²/+ ♀♀	75	69.44 ± 1.42	12.34		3–7	3–8	3–10
5	DCBA	ey²/+e/+ ♀ X rl/+se/+ ♂	ey²/+e/+rl/+se/+ ♂♂	74	65.68 ± 1.47	12.68	70.19	4–8	4–9	
6		(DC) (BA)	ey²/+e/+rl/+se/+ ♀♀	70	74.71 ± 2.06	17.30		5–6	5–8	4–10
7	BADC	rl/+se/+ ♀ X ey²/+e/+ ♂	rl/+se/+ey²/+e/+ ♂♂	71	69.46 ± 1.48	12.51	72.98 (69.51)	6–9	6–10	5–10
8		(BA) (DC)	rl/+se/+ey²/+e/+ ♀♀	62	76.50 ± 1.30	10.28		7–8	7–9	7–10
9	K	Hacettepe w.t	(Control) ♂♂	85	62.72 ± 1.41	13.06	59.68	8–9	8–10	
10			♀♀	75	56.64 ± 2.06	17.29				

Control group is the same as in Table 1 and Table 2.

the duration of adult-life. Each of the 4 mutants (*rolled*, *sepia*, *ebony* and *eyeless*) studied has a specific mean life-span and the expression of these mutations is sometimes sex-dependent. Although not significant, *sepia* males have longer adult life than the respective females, and there is similarity between the means of the sexes of *rolled*, and of *ebony*. The *eyeless* males have a significantly longer mean duration of life than the females. The present results concerning sex difference in life-span are in accordance with the previous reports of, for example, Woodhams and Hollingsworth (1971), Lints (1971) and Bozcuk (1978), that there is no consistent pattern with regard to sex differences. Indeed, as stated

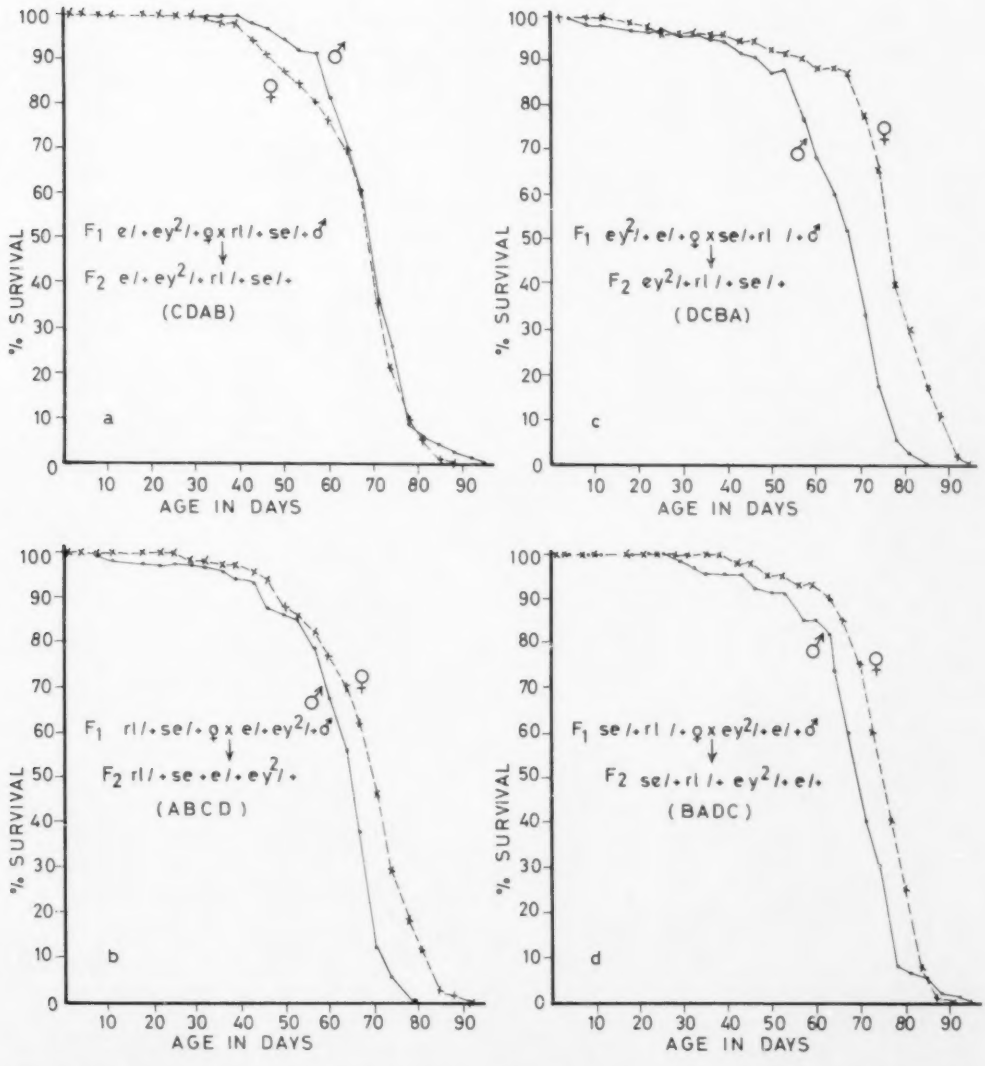

FIG. 3. Survival curves for the F_2 hybrid progenies produced by the reciprocal crosses of the F_1 heterozygotes.

later by Parsons (1978) "sex differences occur favouring one or the other of the sexes in different strains."

The sex-combined mean life spans of *rolled*, *ebony* and *eyeless* inbred lines are significantly shorter than the w.t. controls. Shortened life-span of mutants has been known since Gonzalez (1923) and this topic has been reviewed recently by Lints (1978 and 1980). In this connection Lints (1980), in his recent review, points out that "major mutations influence life-span in a negative way i.e., they shorten it." According to Parsons (1978) there are genes with major morphological, physiological and behavioral effects having associated effects on life-span. In fact, these mutations may alter the total developmental picture, as well as enzymic functions, so that they result in the reduced duration of life of flies.

We may note in passing that Biscardi and Webster (1977), found a mean of 36 days for their *ebony*, mutant flies, where as it is around 46 days in the present work. However the food used, population density and the method of transfer on to fresh medium differed from ours, and this difference of technique may well have influenced the results.

The F_1 generation produced by the reciprocal crosses between *rl* and *se*, *e* and ey^2, do have a prolonged mean life span compared with both the mutant parents and the w.t. controls (Table 2 and Fig. 2). The extension of life span of F_1 hybrids is about 35.5% against mutant parents and 21.7% against w.t. controls. The prolongation is more pronounced in the F_1 of ey^2 ♀ × *e* ♂ and *se* ♀ × *rl* ♂, the reason for which is not clearly understood. In the latter type, the prolongation of life span for F_1 females is more than for males (the difference is significant), but there is no such difference between the F_1 sexes of the former group.

When depressed inbred lines are crossed together, the F_1 hybrids exhibit heterosis for longevity (Clarke and Maynard Smith, 1955; Lints, 1978; Comfort 1978). Heterosis is a normal phenomenon and expected in crosses among inbred strains, even if these strains are mutants (Clark and Gould, 1970; Bozcuk, 1978; Ünlü and Bozcuk, 1979a). There is also a recent work reported by Bozcuk *et al.* (1979) that a kind of heterosis can even be produced between two *vestigial* stocks if these were reared at different *Drosophila* centers for a long time.

In the present findings, in general, heterosis is greater in females than males as is seen Tables 2 and 3, Here, the averages for females in the F_1 and F_2 generations are respectively 68.03 and 72.13 days, whereas for the males they are respectively 64.61 and 67.03 days. On the other hand, for the mutant parents the averages were 47.1 days for the females and 50.7 days for the males. This result is confirmed by the work of Parsons (1966) in which heterosis in crosses among inbred strains is usually greater in females than males. At the same time, in the same work, heterosis was found to be more extreme at 29.5°C which is a very unfavourable environment for *D. melanogaster*. In *Drosophila* crossing inbred strains in several species often leads to prolonged duration of life and heterosis, which is magnified under extreme temperatures (reviewed in Comfort, 1978).

It was also desired to test the possibility of modifying the reduced adult life span of the mutants by selective crossing and further hybridisation. The data in Table 3 and Fig. 3 show generally that the life of adults are lengthened by further (F_2) hybridisation. The sex-mixed overall mean of the F_2 was 42% increased relative to mutant parents, which is more than the increase observed in the F_1 hybrids.

It is seen in Table 3 that the means for the females of ABCD, DCBA and BADC are greater than for their respective males, but that they are similar in the CDAB group. The

average life-spans for the females and males of the F_2 generation are respectively 72.13 and 67.03 days, which exceed the respective means both in the F_1 and P generations. While only the males of *eyeless* have a significantly greater mean than that of the corresponding females, in the BA group (se/+ rl/+) of the F_1 generation the females are the longer-lived sex. When we examine the situation in the F_2 it will be seen that, except for the CDAB group in all 3 other groups females have larger means than their respective males. This is in contrast with the findings of Clarke and Maynard Smith (1955) namely that sex differences in the hybrid F_1 populations of *D. subobscura* are less marked than for the w.t. parents. It is not known if this is due to the different species or to some other factor.

If we calculate the Parsons' (1966) measure of heterosis $(\overline{F} - \overline{P}) / (\overline{F} + \overline{P})$, where $\overline{F}$ and $\overline{P}$ are respectively the overall survival times of hybrids and inbred parents, for the data in this study it will be seen that there is hybrid vigour in the F_1, which is not diminished in the F_2. Although there is insufficient data to draw a general conclusion, it can be said that the measure of heterosis is not dependent in a simple way on whether the parents are w.t. or mutant, or upon the generation at which heterosis is measured. Indeed, the F_2 generation in the work of Woodhams and Hollingsworth (1971) and in the present paper have larger measure of heterosis than the corresponding F_1, whereas in the case of Ünlü and Bozcuk (1979a) it was less in the F_2 than F_1. This may arise either from the fact that in the F_2 there is a heterozygosity for some gene orders or that new but unknown types of interactions arise in the F_1 and F_2 generations (Bozcuk, 1978).

Vol. 16 1981

It is also observed in this study that duration of adult life is associated with certain mutant genes controlling morphological characters such as wing shape, eye colour, body colour, and eye size. This might arise from the fact that each mutations might also have pleiotropic effects reducing longevity. Hybridisation among these mutant lines helps to cure the detrimental effects of the mutation on both the F_1 and F_2 populations, due to heterosis (hybrid vigour).

According to von Hahn (1973) there are two types of hypothesis in the "genetic program" of ageing. The first type states that the ageing process is really a continuation of growth, differentiation and morphogenesis, and not a special or different biological phenomenon. There is little evidence to support this view at the moment (Lints and Soliman, 1977). The second type states that there exists a distinct group of "ageing genes" which are not functional during growth and development but which are activated once adult homeostasis and the end of the reproductive period have been reached. These "senescence genes," that have somehow, either by natural selection or by random drift processes been inaccessible to natural selection (Medawar, 1957), have accumulated in the genomes. Sacher (1978) examining the "senescence gene theory" thinks that there is no evidence for the existence of senescence genes, which are postulated to have benign expression in early life and then to switch to a deleterious senescent expression in later life. He also adds that if ageing is the result of an unknown number and variety of senescence genes, then there is no feasible program for their identification and removal, and therefore offers no hope of significant advance beyond our present condition. On the other hand, Lints (1980) in his recent review argues that "the impossibility to select towards a higher longevity and the total absence of relation between parental and offspring longevities demonstrate that the very large phenotypic variability displayed by longevity in wild strains of *Drosophila melanogaster* does not depend on a precise set of specific genes or polygenes with additive action." In continuing he adds that there is no mutation to pro-

long life span, therefore evidence from major genes is not conclusive in favour of specific "longevity genes." Recently, a third possible hypothesis has been added to the previous two by Sacher (1978) which is called by him "longevity-assurance genes." He claims that this hypothesis, appears to be more consistent with evolutionary and genetic theory, and as a support for the hypothesis he quotes the results of Hart and setlow (1974). In addition, acceptance of the hypothesis of longevity-assurance genes implies that by developing some means, our evolved and genetically controlled longevity-assurance mechanisms could be manipulated.

Although currently fruitful, genetic studies of ageing need more basic data in order to understand clearly the genetic program of ageing. Many problems of genetic engineering will have to be solved before ageing can be retarded and life-span in human beings significantly prolonged.

SUMMARY

The specific effects of four autosomal mutant genes (*rl*: 2–55.1; *se*: 3–26.0; *e*: 3–77.7 and ey^2: 4–2.0) upon adult longevity have been measured. The overall average life span of these mutants (49.94 days) was 17.99% shorter than the Hacettepe w.t. strain (59.68 days). The F_1 generation of the reciprocal crosses between *e* ♀ × ey^2 ♂ and *rl* ♀ × *se* ♂ has an average life-span (66.32) increased by 35.51% and F_2 offspring (69.51) has an extension of 42%. Both the maximum longevities and heterogeneity are increased in F_1 while only homogeneity is increased in the F_2. Heterosis in crosses among inbred strains is greater in females than males. Sex differences in the hybrid populations are more marked than the inbred parents. The detrimental effects of the mutants are cured by hybridisation both in the F_1 and F_2 due to heterosis. The results are discussed from the point of view of a genetic program of ageing.

Acknowledgements—I wish to thank Dr. Zehra Muluk for the interest she has shown in the statistical data, Mr. L. M. Brown for reading the manuscript, Naci İleri, Mustafa Bütüner and Gülbahar Meşeci for technical assistance.

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Vol.
16
1981

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Vol.
16
1981

ANNOUNCEMENT

Invitation

10TH EUROPEAN CONGRESS OF CLINICAL GERONTOLOGY OF THE INTERNATIONAL ASSOCIATION OF GERONTOLOGY

Date and Place of the Congress: September 1–3, 1983, Budapest, Hungary

Main Topics of the Congress

1. Clinical consequences of longitudinal studies in the aged
2. Metabolic disorders in the aged
3. Bone and joint diseases in the elderly
4. Free papers

Scientific Correspondence: Gerontology Center Semmelweis
Medical School
Budapest, Somogyi Béla u 33.
H-1085

Detailed Information From: CONGRESS BUREAU MOTESZ
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ERRATUM

Readers are advised that through a production error, the paper entitled "Age-Related Changes in Liver Drug-Metabolizing Enzymes," by D.L. Schmucker and R.K. Wang, was published in both Vol. 15, No. 4 (pp. 321–329) and Vol. 15, No. 5 (pp. 423–431). The version published in Vol. 15, No. 5 represents the authors' final revisions: it alone should have been published. The publisher regrets the error and apologizes to both the authors and the readers for the duplication.

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SERUM PROFILES OF LH, FSH, TESTOSTERONE AND 5α-DHT FROM 21 TO 1000 DAYS OF AGE IN *AD LIBITUM* FED AND DIETARY RESTRICTED RATS

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Vol.
16
1981

INTRODUCTION

DIETARY RESTRICTION instigated immediately postweaning remains one of the most effective means of modifying early development and the apparent rate of rodent senesence, (McCay, 1952; Ross and Bras, 1965; Berg and Simms, 1961; Miller and Payne, 1968; Ross *et al.*; 1976). Dietary restricted animals have decreased incidence and delayed onset of many chronic diseases, specifically chronic pneumonia, nephrosis and tumours (Ross, 1964). Basic data on this model system is limited and the operative effect is unknown. Samuels (1946) and Segall (1979) have suggested that the effect is mediated by the pituitary in the form of a dietary hypophysectomy with the pituitary remaining dormant whilst the animal is maintained on a restricted diet, followed by reactivation on return to *ad libitum* feeding.

The interrelation of pituitary gonadotrophins and testicular androgens during development and maturation of the male reproductive system and initiation of spermatogenesis has been well documented (Mackinnon *et al.*, 1978; Swerdloff, 1978). Serum concentrations of prolactin, FSH and LH during development have been reported by several groups (Ojeda and Ramirez, 1972; Döhler and Wuttke, 1975; Mackinnon *et al.*, 1976; Piacsek and Goodspeed, 1978), whilst the effects of ageing on the reproductive system have been studied by Adams (1972), Riegle and Miller (1978) and Saksena and Lau (1979). Eleftherion and Lucas (1974) and Nelson *et al.*, (1975) observed no change with age in serum testosterone in the mouse but Chan *et al.* (1977) and Ghanadian *et al.*, (1975) reported a significant decline in serum testosterone with age in the rat which is associated with decreased testicular Δ^5-3β-hydroxysteroid dehydrogenase activity, (Leathem and Albrecht, 1974, Collins *et al.*, 1972). In the rat, Riegle *et al.*, (1977) have observed with age both a decreased pituitary content of luteinising hormone (LH), and a reduced pituitary responsiveness to LHRH associated with an altered hypothalamic sensitivity to steroid feedback (Riegle and Miller, 1978; Miller and Riegle, 1978). Lowered serum concentra-

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tions of LH and FSH have been reported by Bruni *et al.*, (1976) in aged rats in response to single or multiple injections of gonadotrophin releasing hormone.

The detrimental effect of underfeeding on reproductive function in adult male rats with associated reduction in acccessory organ weights and reduced serum levels of testosterone (Grewal *et al.*, 1971), is well documented. Spermatogenesis appears to be little affected by underfeeding for 50% feed restriction for 23 weeks had no significant effect on epididymal or testicular sperm concentrations (Grewal *et al.*, 1971). Howland (1975) reported reduced serum levels of LH in underfed adult male rats but neither serum or pituitary concentrations of FSH were influence by the feed level. Rats undernourished for 20 days exhibited lowered serum testosterone.

Within this context it seemed pertinent to establish the lifespan serum profiles for both pituitary gonadotrophins and testicular steroids in fully fed and dietary restricted animals and to relate these to both the timing of sexual maturation and the maintenance of fertility.

METHODS AND PROCEDURES

Animals

Male Sprague-Dawley (CFY strain) rats were weaned at 21 days and randomly allocated to one of three groups. In group (G) animals were fed *ad libitum* and housed 4 animals to a cage. Group (I) animals were fed *ad libitum* but housed individually. Group (DR) animals were caged individually and were dietary restricted. Animals fed *ad libitum* were allowed unlimited access to a pelleted diet, (Rat Breeder diet, E.H. Bradshaw & Sons, Ltd., Driffield, England), whilst growth was retarded in the dietary restricted animals by limiting intake of the diet such that body weight was maintained at 50% that of the *ad libitum* fed individually housed group. The restricted diet was supplied at 1100hrs colony time each day. All animals had unrestricted access to water and the temperature was maintained at 22∓ 1°C, humidity within the range 50–70%, and a light regimen of 12 hrs light/24 hours. Body weight was measured throughout life in 397 group (G) rats, and 100 group (I) and group (DR) rats. Cumulative mortality was recorded and expressed in 50 day cohorts for each of the three groups of rats. Animals used to establish hormone profiles or breeding performance were matched to these growth curves. Serum Hormones: Serum concentrations of LH, FSH, testosterone and 5α-dihydrotestosterone were determined at regular intervals from weaning to 1000 days in each of the three experimental groups. Blood (3.5 ml) was removed from the infraorbital sinus of rats lightly tranquillized with 1:120 (v/v isotonic saline) dilution of Immobilon ® (Reckitt & Colman Ltd., Hull, England) and the sera obtained stored at −25°C until assayed. Animals were bled between 14.00 and 15.00 hrs colony time and were sampled only once.

LH and FSH concentrations in 0.2 ml samples were measured by the double antibody technique of Niswender *et al.*, (1968) using materials generously supplied by the NIAMD Rat Pituitary Program, Bethesda, U.S.A. Purified rat LH or FSH was labelled with ^{125}I using 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril, (Pierce Chemical Co., Rockford, USA). All samples were measured in duplicate with an intra-assay variance of 7.7% (LH), 7.9% (FSH) and an inter-assay variance of 10.0% (LH) and 9.1% (FSH). Results are expressed as nanograms of NIAMD-ratLH-RP-1 or NIAMD-ratFSH-RP-1 per ml.

Testosterone and 5α-dihydrotestosterone concentrations were determined in 0.4 ml serum aliquots. To each aliquot 1000 cpm of ^{3}H-testosterone (sp.act. 100 Ci/mMole) and 1000 cpm ^{3}H-5αDHT (sp.act. 101Ci/mM) were added and the samples diluted to 1 ml with water. The steroids were extracted into 2 × 3 ml of peroxide free diethyl ether, the extracts pooled and evaporated to dryness at 40°C under N_2. The concentrated extract was chromatographed on an LH-20 column in isooctane, benzene, methanol, (90:5:5 v/v) to separate testosterone and 5α-DHT according to the method of Auletta *et al.*, (1979). Testosterone and 5α-DHT were assayed with an antisera raised in rabbits against testosterone-7α-BSA (Miles Laboratories Ltd., England), which had a cross-reactivity to 5α-DHT of 40%. The assay buffer was 0.05M tris-HCl (pH 8.0) containing 0.15M NaCl, 0.1% sodium azide and 0.1% gelatin. Column fractions containing testosterone and 5α-DHT were dried down and redissolved in 0.75 ml of assay buffer. An aliquot of 0.15 ml was taken to determine procedural losses, the calculated recovery for testosterone was 47.7 ∓ 0.7% (s.e.m, n = 178) and 5α-DHT 41.9 ∓ 1.0% (s.e.m, n = 174). Duplicate aliquots of 0.25 ml were taken for mass determinations. Standards (testosterone; range 0.01–1.0 ng/0.1 ml: 5α-DHT, range 0.01–0.5 ng/0.1 ml) and sample aliquots were made up to 0.3 ml with assay buffer. To all tubes, excluding the non-specific blanks, to which 0.5 ml of assay buffer was substituted, 0.5 ml of antisera was added. After 30 mins preincubation at room temperature (22°C), 10,000 cpm of ^{3}H-testosterone or 5α-DHT in 0.1 ml assay buffer was added to all tubes, mixed and incubated at 37°C for 1 hour, followed by a

further incubation at 4°C for a minimum of 2 hours. Antibody bound and free hormone were separated by the addition of 0.2 ml of 0.5% w/v charcoal and 0.05% w/v dextran T-70 suspension. After incubation at 4°C for 15 mins, the charcoal was precipitated by centrifugation at 1500g for 15 mins. The supernatant was decanted into scintillation vials to which 8 ml of emulsifier 299 (Packard Instruments, Illinois) was added. Samples were counted to an error of less than 2% and the minimum detectable dose per tube was 0.005 ng of testosterone and 0.010 ng of 5α-DHT, with an intra-assay variance of 6.4% (testosterone), 7.0% (5α-DHT) and inter-assay variance of 11.8% (testosterone) and 9.5% (5α-DHT).

Fertility was assessed in virgin group (I) and (DR) rats at the following ages; 63–84 days, 107–130 days, 400–530 days, 660–760 days. Within each age range 10 rats from each group were caged with 3 randomly selected virgin 3 month old female rats during the period 1700–0900 hrs colony time each day for 15 days. During the period 0900–1700 hrs the male rat was returned to its own cage to facilitate feeding the allotted diet. No food was assigned to any animal from 1700–0900 hrs each day. The appearance of vaginal plugs was recorded and after 15 days each female was individually caged. Fertility in each male was denoted by the siring of one or more litters.

Statistics

Body weight data were tested for significant difference with the Wilcoxon test for two samples, ranked observations, not paired. Cumulative mortality curves were analysed by probit transformation. The significance of the difference between means of serum hormone concentrations was tested by analysis of variance and the Student's test: significant covariance of two hormones was shown by correlation analysis. Data from the breeding studies were analysed by a 2 × 2 contingency table using the chi-squared test, (Sokal and Rohlf, 1969). Radioimmunoassay and quality control data were processed by computer programmes supplied by the National Technical Information Service, US Department of Commerce, according to the criteria proposed by Rodbard and Frazier (1972) and Rodbard *et al.* (1975).

Vol. 16 1981

RESULTS

Growth curves, mortality profiles

Growth curves from 21 to 1000 days for the three groups of rats are shown in Fig. 1. Between 40 to 50 days a significant ($p < 0.001$) slower gain in body weight is distinguishable for the *ad libitum* fed individually housed rats compared with those held in groups. A maximum weight of approximately 700 gms is seen at 400–500 days in individually housed animals whereas grouped rats reached a maximum body weight of 900–1000 gms at 700 days following which a rapid loss of weight was observed. Animals held on a restricted diet were allowed to grow steadily and reached a peak weight of 400 gms at 900 days after which a slow but progressive loss of body weight was observed. Cumulative mortality expressed as a percentage is plotted in 50 day cohorts for the three groups of rats (Fig. 2). Although a significant slower rate of growth was observed in individually housed *ad libitum* fed rats when compared with those held in groups of four, no significant difference was recorded in the mortality profiles. The LD_{50} recorded for *ad libitum* fed animals was 694 days with a maximum lifespan of 1056 days. Dietary restriction had the effect of delaying the increase in rate of mortality from 500 to 600 days. Probit analysis gave an LD_{50} of 988 days and the maximum lifespan in the group was 1500 days.

Serum hormone profiles

In all groups serum testosterone concentration was low (0.2 – 0.5 ng/ml) between 21 and 35 days, (Fig. 3, Fig. 4, Fig. 5). A significant rise in testosterone concentration was observed in the two *ad libitum* fed groups between 35 and 40 days reaching a peak of 5–6 ng/ml at 60 to 70 days, after which age serum testosterone declined rapidly reaching 1–2 ng/ml by 100–150 days. The *ad libitum* fed grouped rats showed a steady decline of serum testosterone from 100 to 1000 days, (1.5 – 0.3 ng/ml) whilst the individually housed *ad*

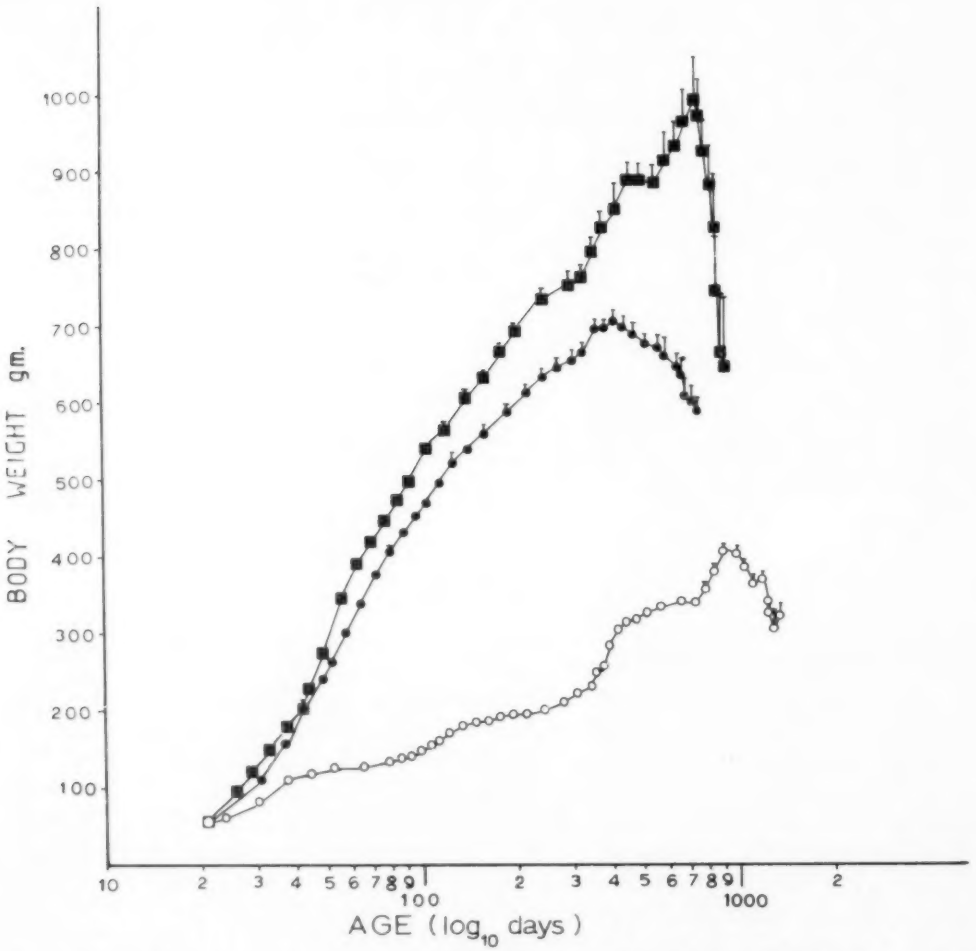

FIG. 1. A comparison of growth curves throughout life for groups (G) = ■; group (I) = •; and group (DR) male rats = ○. Initial group numbers, group (G) = 397, group (I) = 100, group (DR) = 100. The s.e.m. is shown when it exceeds the width of the symbol.

libitum fed rats exhibited a decline in serum testosterone only between 450 and 1000 days. A significant difference ($p < 0.05$) is seen in serum testosterone levels of grouped and individually housed *ad libitum* fed animals between 450 and 1000 days. A rise in serum testosterone concentrations was not observed in the dietary restricted animals until 50 days and the peak values were not recorded until 70 to 80 days. Peak values for serum testosterone concentrations were significantly greater ($p < 0.001$) in *ad libitum* fed rats compared to those on a restricted diet. The rapid decline in serum testosterone concentrations observed in *ad libitum* fed animals between 90 and 100 days was also seen in the dietary restricted group although by 140 days a second peak of serum testosterone was recorded. Between 200 to 1000 days in dietary restricted animals serum testosterone remained at approximately 1.0 ng/ml (Fig. 5).

5α-DHT serum levels in the *ad libitum* fed grouped rats were approximately 200–300

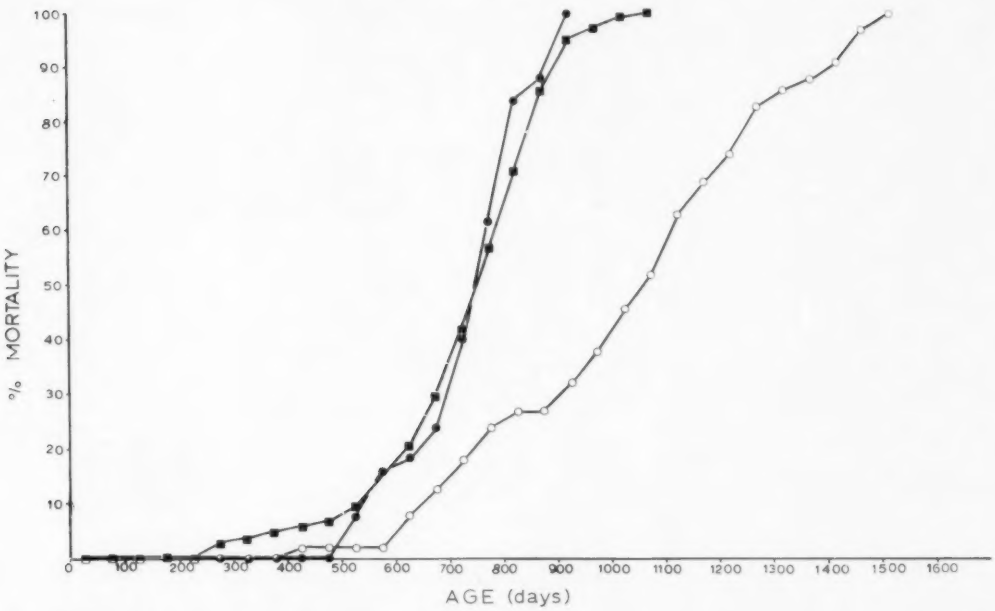

FIG. 2. Cumulative mortality profiles plotted in 50 day cohorts for group (G) = ■; group (I) = •; and group (DR) male rats = ○. Initial group numbers, group (G) = 397, group (I) = 100, group (DR) = 100.

pg/ml between 21 and 40 days then steadily declined to 100 pg/ml by 100 days, after which age 5α-DHT levels remained at approximately 100 pg/ml (Fig. 3). In individually housed *ad libitum* fed animals a rapid decline in 5α-DHT was observed between 21 and 30 days (300 to 100 pg/ml) after which age the levels remained relatively constant throughout life at 100–200 pg/ml (Fig. 4). Serum 5αDHT concentrations in dietary resticted rats were 200–300 pg/ml from weaning to 40 days then steadily declined to 100 pg/ml by 250 days, remaining at this level until 1000 days. The decline in serum 5α-DHT concentrations in dietary restricted rats with age was similar to that observed in the grouped *ad libitum* fed animals but occurred at a slower rate.

A sharp rise in serum FSH concentrations was observed in all groups between 21 and 30 days reaching a peak of 600–700 ng/ml (Figs. 3, 4, 5). In *ad libitum* fed rats high serum FSH levels were maintained until 35–40 days after which age the values fell rapidly to reach a nadir of 70–120 ng/ml at 70–80 days. After 80 days in the grouped housed *ad libitum* fed rats, serum FSH concentrations increased to plateau at approximately 300 ng/ml until 1000 days. Between 80 and 1000 days grouped *ad libitum* fed rats had significantly higher serum FSH ($p < 0.001$), than *ad libitum* fed rats which were individually housed (Fig. 3, Fig. 4). A significant negative correlation of serum FSH and testosterone concentrations ($p < 0.01$, $r = -0.53$) and a significant positive correlation of serum FSH and 5α-DHT ($p < 0.01$, $r = 0.65$) is seen in grouped *ad libitum* fed rats, between 21 and 100 days. Serum FSH concentrations of dietary restricted animals showed a precipitous fall after 30 days reaching 80 ng/ml at 45 days with a subsequent rise to 100–200 ng/ml between 50 and 80 days. After 100 days serum FSH levels in this group rose steadily until 1000 days (Fig. 5).

Vol. 16 1981

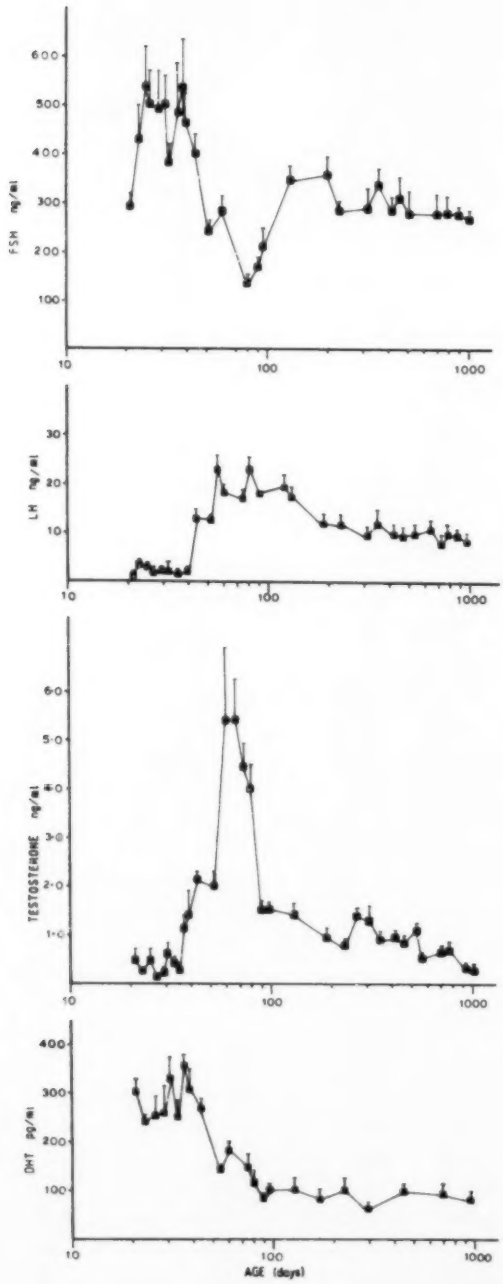

FIG. 3. Serum profiles of FSH, LH, testosterone and 5α-DHT from 21 to 1000 days in grouped *ad libitum* fed male rats. Each point is the mean and s.e.m. for 10 animals.

Vol. 16 1981

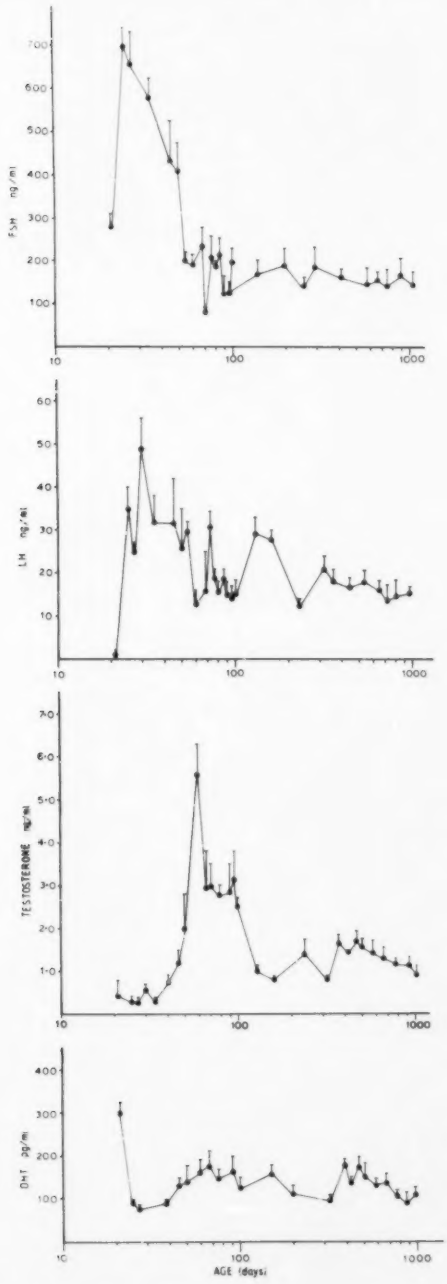

FIG. 4. Serum profiles of FSH, LH, testosterone and 5α-DHT from 21 to 1000 days in individually caged *ad libitum* fed male rats. Each point is the mean and s.e.m. for 10 animals.

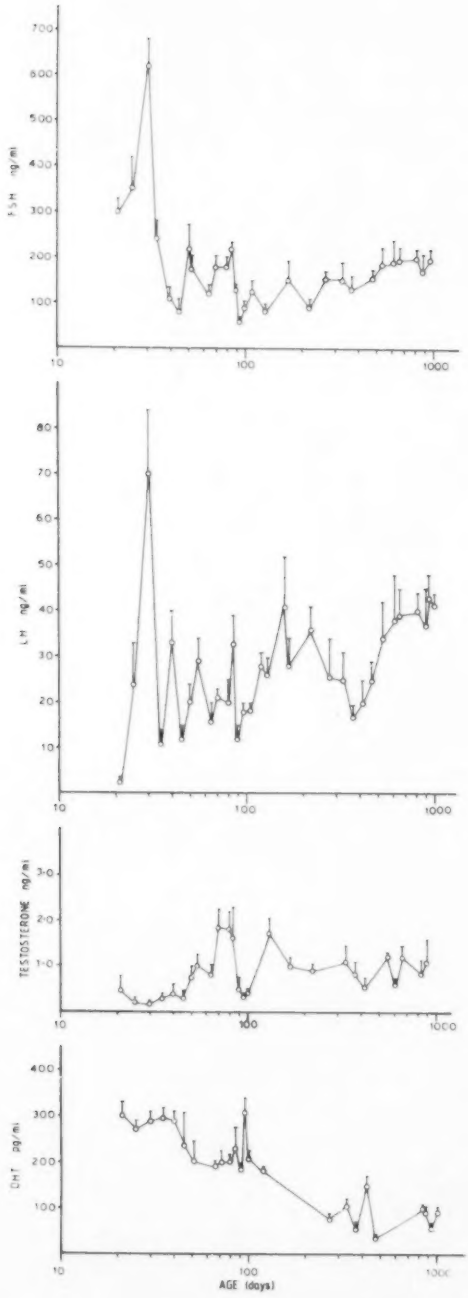

FIG. 5. Serum profiles of FSH, LH, testosterone and 5α-DHT from 21 to 1000 days in individually caged dietary restricted male rats. Each point is the mean and s.e.m. for 10 animals.

In grouped *ad libitum* fed animals serum LH was very low between 21 to 40 days (2–4 ng/ml), approaching the limit of sensitivity for the assay. After 40 days serum LH concentrations rose sharply to peak values of 20 to 25 ng/ml between 55 and 85 days, after which age serum LH values declined with age (Fig. 3). A significant positive correlation of serum LH and testosterone is demonstrable between 21–1000 days of age in grouped *ad libitum* fed animals ($p < 0.01, r = 0.77$). Higher serum LH levels were observed in individually housed *ad libitum* fed animals between 25–70 days of age when compared with those held in groups (Fig. 4). Only between 70 and 100 days were LH serum values similar in the two *ad libitum* fed groups (15–20 ng/ml). In the individually housed animals a steady decline in serum LH concentrations was recorded between 120 and 1000 days. Marked instability and individual animal variation of serum LH concentrations was recorded in animals on the restricted diet (Fig. 5). Serum LH levels increased rapidly from 21 to 30 days reaching a peak of 70 ng/ml at 30 days, after which a precipitous fall was observed followed by a series of short term oscillations, the trend of which showed a steady elevation of serum LH with age. At 90 days a sharp fall in serum LH concentrations was observed which corresponded with a fall in serum testosterone (Fig. 5). Fertility studies: There was a significant delay ($p < 0.005$) in sexual maturation of the dietary restricted animals compared with the individually housed *ad libitum* fed rats (Table 1). Only 30% of dietary restricted animals sired a litter in the 63 to 84 day cohort compared to 90% in the fully fed animals. At the other age cohorts where fertility was assessed no significant difference was distinguishable between groups. The quotient of number of litters sired to maximum number of litters possible reveals a significant difference between groups at three of the four age ranges tested (Table 1).

Vol. 16 1981

DISCUSSION

Serum testosterone levels observed in *ad libitum* fed rats either individually housed or caged in groups of four confirmed the developmental profiles reported by de Jong and Sharp (1977), and Saksena and Lau (1979). Whilst a number of authors have published data for serum or plasma testosterone concentrations in young, adult and old rats, only Saksena and Lau (1979) have reported a detailed and systematic study of hormone profiles with age in the male rat. However these observations were terminated at 450 days which excludes the period of senescence in most strains of rat. A steady decline in serum testosterone with age was observed in fully fed rats in agreement with the findings of Ghanadian *et al.* (1975), Chan and Leathem (1977), Harman, *et al.* (1978). In singly caged *ad libitum* fed rats no consistent decrease in serum testosterone values was observed until after 350 days when the profile of declining serum testosterone was similar to, but, with values significantly higher than in the grouped animals. Various workers have concluded that individual housing constitutes a stressful experience in the rat (Baer, 1971) and the higher serum testosterone concentrations recorded in the individually housed rats after 350 days may reflect increased adrenocortical activity. However in a recent review of the effects of differential housing in the laboratory rat, Brain and Denton (1979) conclude that there is considerable conflicting data on this point which is difficult to interpret.

The suppression of serum testosterone by feed restriction has been reported for adult rats by Howland (1975) and Grewal *et al.* (1971). In the present work rats dietary restricted from 21 days showed both a delay in the timing of the pubertal peak of serum testosterone and a marked suppression of the peak height. During development in fully

TABLE 1. EFFECT OF CHRONIC DIETARY RESTRICTION ON BREEDING PERFORMANCE IN MALE RATS OF DIFFERENT AGES

Age range	No. males siring 1 or more litters (Tot. no. males tested in brackets)		No. males siring litters expressed as a quotient		Total no. litters sired (maximum possible no. litters)		Total no. litters sired expressed as a quotient of maximum possible	
	Isolate	Dietary restricted	Isolate	Dietary restricted	Isolate	Dietary restricted	Isolate	Dietary restricted
63–84	9[10]	3[10][c]	0.90	0.30	23[30]	4[30][c]	0.77	0.13
107–128	9[10]	9[10]	0.90	0.90	25[30]	15[30][b]	0.83	0.50
400–530	9[10]	8[10]	0.90	0.80	24[30]	19[30]	0.80	0.63
660–760	5[10]	6[20]	0.50	0.30	10[30]	8[60][a]	0.33	0.13

[a] $p = 0.05$, between isolate and dietary restricted animals at a given age range; x^2, 2×2 contingency table.
[b] $p = 0.01$
[c] $p = 0.005$.

fed rats, enhanced testosterone synthesis precedes spermatogenesis whilst restriction of food is reported to delay the onset of puberty (Leathem, 1970; Piacsek and Goodspeed, 1978). This observation is supported by the comparison of fertility and breeding performances of the dietary restricted rats with fully fed animals in the 63–84 day age cohort. In those dietary restricted males which were fertile at this age, poor breeding performance was recorded and this may reflect both qualitative and quantitative changes in the ejaculate. No significant difference in fertility was observed after 100 days between fully fed and dietary restricted rats whilst serum testosterone values in the dietary restricted animals were similar to those in the fully fed animals. In contrast to *ad libitum* fed animals no decline in serum testosterone concentrations was observed with age in this group.

Howland (1975) considered that underfeeding in the adult rat affected the androgenic function of the testes more than the spermatogenic function. In the present study this conclusion is confirmed only for the first 100 days postweaning. However at all age ranges tested except 400–530 days, a significant reduced ability to sire multiple litters is seen (Table 1). Amino acid deficiencies interfere with testis maturation in a manner not entirely due to inanition (Erschoff, 1952). Deficiencies of arginine (Shettles, 1960), lysine and tryptophane (Leathem, 1970), valine (Scott, 1964) and methionine, (Pomeranze *et al.*, 1959) are all reported to interfere with spermatogenesis. Adult rats fed a poor protein such as maize or gelatin at 15–18% of the diet show decreased sperm motility and increased number of abnormal sperm which may be a response to deficiencies of individual amino acids (Leathem, 1970).

Vol. 16 1981

There is considerable disagreement between published data of serum LH values during development in the rat. The profile observed in the present work for the grouped *ad libitum* fed animals is in agreement with the report of Piacsek and Goodspeed (1978) and Saksena and Lau, (1979) although the high LH levels reported by these authors at 21 days were not observed. The significant correlation of serum LH and testosterone observed throughout life in the grouped *ad libitum* fed animals may well suggest a hypothalamic-pituitary origin of the age related serum decline of testosterone, and is in agreement with the findings of Miller and Riegle (1975). The wide fluctuations in serum LH observed during development which were reported by Piacsek and Goodspeed (1978) and MacKinnon *et al.* (1978) were not observed in the grouped *ad libitum* fed animals, however, in rats caged individually high levels of serum LH were recorded. Restricted feeding which resulted in very high prepubertal LH values may result from the severe stress that this regime imposes. Döhler *et al.* (1977) have shown that stress increases serum concentrations of LH in the rat.

The developmental profiles of FSH in serum recorded between 21 and 100 days in both grouped and individually housed *ad libitum* fed rats are similar too and in agreement with the findings of Swerdloff *et al.* (1971), and Odell and Swerdloff (1976). An initial increase in serum FSH between 20 and 30 days followed by a decline at 40 days is in contrast to the findings of Ojeda and Ramirez (1972), Döhler and Wuttke (1975), and de Jong and Sharp (1977) who did not observe a fall in serum FSH levels until around day 60.

In contrast to the report of Howland (1975) on adult rats restricted to 50% feed, such restriction in prepubertal postweaning rats results in a precipitous fall in FSH levels by 30–40 days of age. Root and Ross (1972) reported significantly lower serum FSH concentrations in prepubertal rats deprived of food for 168 hrs. They considered this was a failure of FSH release rather than synthesis in agreement with Leathem (1966). Stewart *et*

al. (1975) found that immature rats on a 50% restricted diet had decreased serum FSH levels which they presumed was the result of a depletion of FSH-RF (Negro-Vilar *et al.*, 1971).

The hypothesis has been expressed by Odell *et al.* (1974) that sexual maturation is, at least, partly the result of FSH induced testicular sensitivity to LH. Odell and Swerdloff (1976) have suggested that the priming action of FSH on the testes was dependent on the promotion of LH receptors. These authors showed that the lack of responsiveness of the immature testis to LH could be overcome by prior administration of FSH. Their studies indicated that testicular responsiveness to LH progressively increases during maturation and that this responsiveness is conditioned by previous exposure of the immature testes to FSH. In the work of Payne *et al.* (1977) an increased capacity for testosterone synthesis was found after day 32 when serum FSH concentrations were maximal. A very close relationship was observed between the appearance and rate of increase in 17β-hydroxysteroid dehydrogenase and testicular capacity for testosterone synthesis and suggest that the 17β-hydroxysteroid dehydrogenase is a limiting factor in the ability of the testis to respond to LH. Therefore the failure of the testis in the prepubertal individually housed *ad libitum* fed and the dietary restricted rats to respond to the elevated serum LH levels may not be solely explained in terms of LH receptor development. However, the delayed puberty observed in the dietary restristed rats would appear to result from the lowered serum FSH and an inability of the testis to respond to the elevated serum LH. It has been suggested that the process of senescence, like foetal development, growth and maturation may be under similar kinds of neural and endocrine control (Segall, 1979). Controlled growth and sexual maturation is an integral part of the process of differentiation forming part of a programme of gene expression. The imposition of an extraneous growth control through dietary restriction modifies this programme to delay sexual maturation by 10–20 days and extend the maximum lifespan by 42%. There is, however, no associated extension of the reproductive lifespan in the male rat, a finding in direct contrast to our observations in the female CFY rat. Dietary restriction which increased the maximum lifespan by 32% was associated with both delayed puberty (up to 186 days) and a delay in the timing of reproductive senescence from 450 days in female *ad libitum* fed animals to over 900 days in the female dietary restricted rats, (Merry and Holehan, 1979). There is, therefore, a clear sex difference in the modification by dietary restriction of gene expression in the rat as evident by the effect in later life on the timing of reproductive decline. Food restriction clearly modifies the temporal profiles of serum hormones in a manner not compatible with simple hypopituitarism or pituitary dormancy.

SUMMARY

Chronic dietary restriction such that the body weight of CFY Sprague-Dawley male rats was 50% that of animals fed *ad libitum* increased both the average lifespan (LD_{50} 988 days compared to 694 days) and the maximum lifespan (1500 days compared to 1056 days). Serum concentrations of LH, FSH, testosterone and 5α-DHT were measured at regular intervals from 21 to 1000 days in three groups of rats. Group (G) were *ad libitum* fed, caged in groups of four, group (I) individually housed *ad libitum* fed and group (DR) individually housed, dietary restricted animals. Sexual maturation and breeding performance was assessed in groups (I) and (DR) animals in the age cohorts, 63–84; 107–130; 400–530 and 660–760 days.

Serum testosterone concentrations in *ad libitum* fed rats were low prepubertal rising to a peak of 6–7 ng/ml at 60–70 days followed by a rapid fall to 1–2 ng/ml by 100 days. Thereafter a steady fall of serum testosterone concentration was observed with age. Dietary restriction delayed the pubertal peak of serum testosterone by 10–20 days and reduced the peak height from 6–7 ng/ml to 2 ng/ml. No age related decline in serum testosterone was recorded. The serum 5α-DHT profiles in the DR rats was similar to that recorded for group (G) but the pubertal fall in serum 5α-DHT concentration was delayed and occurred at a slower rate. Serum FSH was markedly depressed in the DR rats between 30 and 70 days but was associated with elevated levels of serum LH compared to the fully fed rats. Puberty was delayed 10–20 days by dietary restriction but after 107 days no significant difference was observed in fertility between groups. There was a significant difference in the ability of DR rats to sire multiple litters.

In this study dietary restriction extended somatic lifespan by 42% but no extension of reproductive lifespan was observed.

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Vol. 16 1981

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AGE-RELATED CHANGES IN AMINO ACID POOL SIZES IN THE ADULT SILKWORM, *BOMBYX MORI*

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Vol.
16
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INTRODUCTION

IT HAS BEEN speculated that the aging of an animal upsets the balance between anabolism and catabolism of protein, the ratio of the two decreasing with age. Even though balance is maintained in the majority of proteins in the body, senescence is induced in old animals by the declining of production of some essential enzymes (Du *et al.*, 1977).

Chen (1972) reported that aged males of *Drosophila melanogaster* contained more free taurine, α-alanine and β-alanine than young males. In *Aedes aegypti* β-alanine and taurine were found in low concentrations throughout its lifetime, although total amino acids and α-alanine were remarked to increased with age (Stidham and Liles, 1969). In a living open system, it is generally difficult to detect fine age-related changes in amino acid pools (Burcombe and Hollingsworth, 1970).

Since the silkmoth feeds on nothing after emergence, we can regard it as a biochemically semi-closed system, except for gas exchange, excretion of some meconium at emergence, and oviposition in females (Osanai, 1978). In this closed system amino acid-protein metabolism during aging is carried out only with qualitatively and quantitatively limited materials which were stored in a larval stage by feeding. Under such critical conditions, aged metabolic changes necessary to maintain life would be observed more obviously than in insects with an open system.

MATERIAL AND METHODS

Ripe silkworms of hybrid F_1 Daizo X Hosho were kindly suppiied by professor N. Yoshitake, Department of Sericultural Sciences, Faculty of Agriculture, University of Tokyo. The silkworms were reared at 25°C and fed on mulberry leaves *ad lib.* Treatments after pupation were carried out as described in the previous paper (Osanai, 1978). Freshly emerged adults were kept at 25°C. Mean and maximal life spans were 7.9 ± 5.0 and 17 days in males (n = 180) and 6.2 ± 4.8 and 15 days in females (n = 180), respectively. Imagines from 0- to 12-day-old were sacrified every second day.

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After weighing, one adult insect at each stage was homogenized in 1% picric acid solution with a Potter-Elvehjem-type homogenizer (McGregor and Loughton, 1976). After centrifugation for 10 min. at 27,000 g and 4°C, the obtained yellow supernatant solution was collected. The precipitate was washed once with 1% picrate. The combined deproteinized solution was passed through a column of Dowex 1 × 2 (200–400 mesh, adapted in acetate buffer, pH 7.0) in order to remove picrate. The clear solution was dried *in vacuo* at low temperature. With and without hydrolysis in 6 N HCl at 110°C for 10 hours, the dried material dissolved in redistilled water was analysed on the two-column-one-line system of an amino acid autoanalyser, JLC-6AH® (Japan Electron Optic Laboratory) by developing with both buffer systems composed of lithium and sodium citrate. The amounts of amino acids in the hydrolysed and unhydrolysed parts were compared after chromatography by the amino acid analyser.

RESULTS

1. Identification of ninhydrin-positive substances

Thirty-six free ninhydrin-positive substances were found in the silkworm adult. In Table 1 the substances present are shown with time number. Thirty-five substances were identified by means of 54 species of authentic amino acids and related compounds. Since an unknown substance at peak No. 227 was disappeared when hydrolysed, it may not be an amino acid or amide, but a peptide. A peak of tryptophan was hardly detected in unhydrolysed samples. Cysteine or cystine and β-alanine were mostly not detected. Glutamic acid and aspartic acid were little in amounts, whereas their amides were found in a large quantity in the moths.

2. Age-related changes in free amino acid levels

Age-related, quantitative changes in 22 ninhydrin-positive substances and the total free substances which could be quantitatively determined among 36 are shown in Figs. 1–5. The various changes were divided into groups. Levels of amino acids decreased generally in a few days after emergence with a few notable exceptions. In males lanthionine, cystathionine, phosphoethanolamine and arginine scarcely changed their low levels during the whole adult life.

2-A. Ninhydrin-positive substances increasing with age

Taurine and ammonia increased in amounts with advanced age in both sexes. Level of taurine in males was always higher than in females during the whole lifetime, even just after emergence, when the majority of amino acid levels dropped (Fig. 5). Ammonia held a constant level until middle age but increased at the late stage of adult life (Fig. 2). In this substance there was also sexual difference. Level of ammonia and the increased in old age in males were much smaller than in females, while the beginning of increase in males was earlier. The increased accumulation of toxic substances in body, which could be one of the causes of death in the old age, may be explained by the hypothesis that it becomes difficult at senescence to convert ammonia to inert nitrogen-containing compounds. There is another possibility to explain the large quantity of ammonia determined in the old moth. Ammonia can be sometimes easily released from amides such as glutamine and asparagine. To avoid this possibility preparation procedures containing evaporation were carried out under low temperature and in short time as much as possible. Decomposition of these amides during fractionation on the analyser was also checked, but conversion of amides into amino acids was almost negligible. Histidine in females increased with age,

TABLE 1. NINHYDRIN-POSITIVE SUBSTANCES FOUND IN SILKWORM MOTH AND AUTHENTIC SUBSTANCES USED FOR IDENTIFICATION*

	kynurenic acid
	anthranilic acid
31	o-phosphoserine
39	glycerophosphoryl ethanolamine
42	taurine
47	o-phosphoethanolamine
	urea
131	L-aspartic acid
	hydroxy-L-proline
153	L-threonine
158	L-methionine sulfoxide
165	L-serine
182	L-asparagine
194	L-glutamic acid
	homoserine
198	L-glutamine
	sarcosine (N-methylglycine)
227	unknown substance
	L-α-aminoadipic acid
257	L-proline
262	glycine
274	L-alanine
	L-citrulline
287	DL-lanthionine (bisthioalanine)
297	L-α-aminobutyric acid
321	L-valine
360	L-cystine
386	L-methionine
	argininosuccinic acid
395	L-cystathionine
405	L-isoleucine
413	L-leucine
432	L-tyrosine
465	L-phenylalanine
483	β-alanine
	L-β-amino-iso-butyric acid
	DL-plus allo-hydroxyserine
97	L-ornithine
	N-acetylglucosamine
102	γ-aminobutyric acid
119	ethanolamine
121	ammonium chloride
	N-acetyldopamine
130	L-lysine
141	L-1-methylhistidine
159	L-histidine
	L-3-methylhistidine
	L-anserine (β-alanyl-l-methyl-L-histidine)
	creatinine (l-methylglyco-cyamidine)
	kynurenine
	3-hydroxykynurenine
224	L-carnosine (β-alanyl-L-histidine)
232	L-tryptophan
289	L-arginine

*Ninhydrin-positive substances detected in silkworm moth were marked with time numbers. Acid and neutral substances from kynurenic acid to L-β-amino-isobutyric acid were chromatographed by first column with lithium citrate buffers, pH 2.73, 3.15 and 3.90, and other basic substances by second column with sodium citrate buffers, pH 4.3 and 5.1.

though somewhat irregularly (Fig. 2). This amino acid in males rose in the early adult stage, but dropped a little in middle age, rising thereafter.

2-B. Ninhydrin-positive substances decreasing in the early stages

Valine, isoleucine, leucine, phenylalanine, alanine, glycine, threonine, asparagine, lysine and arginin fell strongly in the early stage, and then kept constant levels or fell a little further in middle age (Figs. 2–4). In the late stage, senescence, the levels again changed. They took a steep increase in 10-day-old adults accompanied with a senescent decline prior to death. According to level change in the adult life these amino acids form a majority group, group M. Perhaps glutamine, proline and methionine belong to this group (Figs. 4 and 5). These three amino acids demonstrated somewhat varied level changes between males and females. Levels of cystathionine, lanthionine and phosphoethanolamine in females changed likewise, though the levels in males were constant throughout imaginal life (Fig. 5). Except for rising after emergence, serine level also changed with the levels of amino acids mentioned above (Fig. 4).

Vol. 16 1981

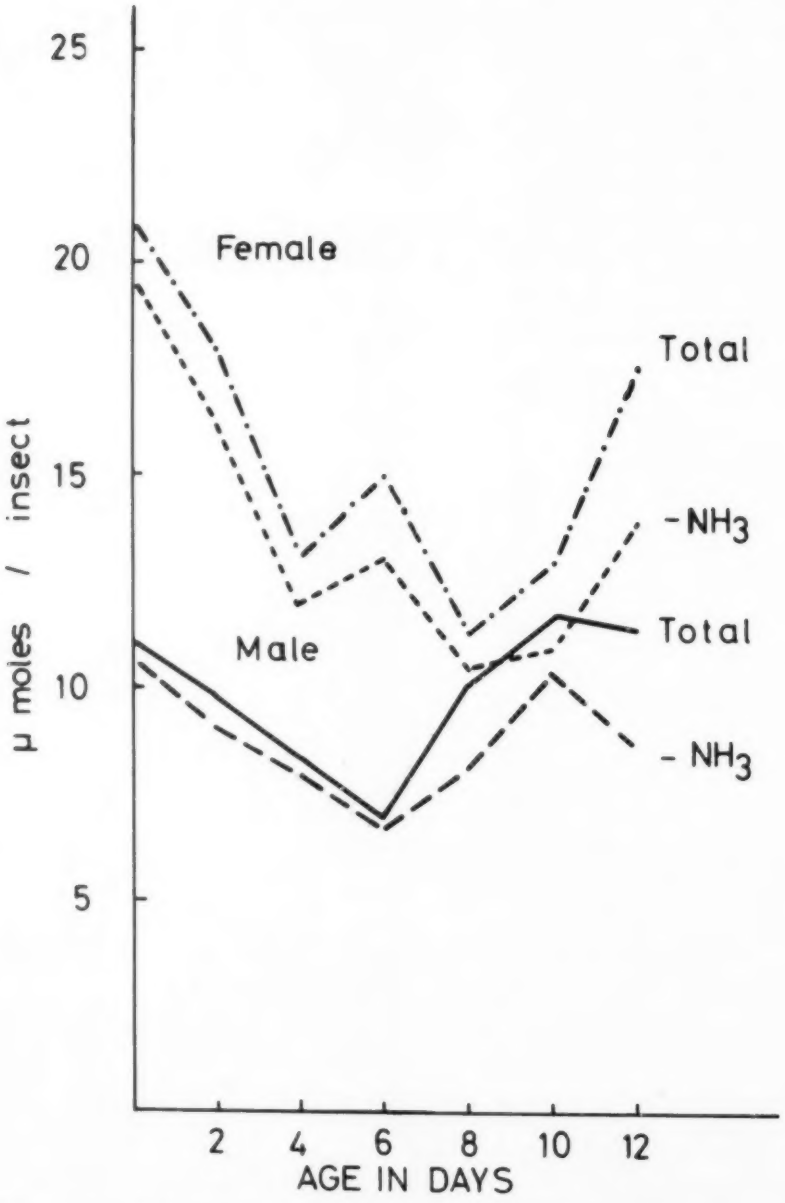

FIG. 1. Changes in the contents of total free ninhydrin-positive substances with or without ammonia during adult lifetime of *Bombyx mori*. Amounts of total ninhydrin-positive substances were calculated as sum of individual substance (see text).

The majority of amino acids and related substances existing in silkworm adults demonstrated a similar tendency of level changes during life. Characteristics of group M were generally presented more distinctly in males than in females, as shown at the peak on the 10th day after emergence. Among 22 ninhydrin-positive substances 14 or 17, if we in-

cluded cystathionine, lanthionine and phosphoethanolamine, demonstrated similar level changes in the lifetime of the silkworm adult. A rapid decrease of amino acid pools in the early stage would be accounted for by a large consumption of amino acids to synthesize protein for postimaginal development after emergence. The pools are stabilized in the middle stage, when the active protein synthesis has apparently finished. The temporary increase of amino acid pools in old age suggest enormous degradation of protein taking place in senescence of this insect.

Level changes in methionine sulfoxide in imaginal life of silkmoth seemed also to be similar to those of group M amino acids, but the distinct peak appeared not on the 10th day after emergence but on the 8th day (Fig. 5). Tyrosine level went down constantly during lifetime (Fig. 4). Especially the decrease in females at the early stage was conspicuous.

2-C. Sexual difference of changes in the amino acid pool

In most amino acids there were large sexual differences of the pool changes during imaginal lifetime. These differences were not only quantitative but also in some substances qualitative.

Amounts of methionine sulfoxide (MSO), taurine, and glutamine in males were through out imaginal life higher than in females (Figs. 4 and 5). On the contrary, 11

Vol. 16 1981

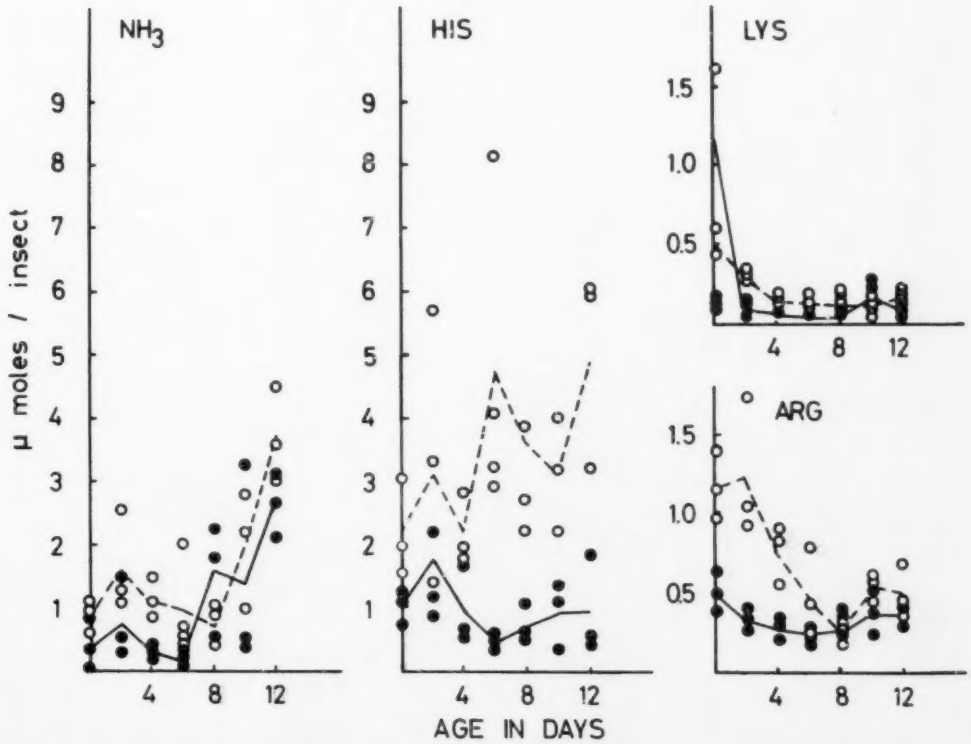

FIG. 2. Changes in the contents of free ammonia, histidine, lysine and arginine during adult lifetime of *Bombyx mori*. (o-------o): females, (•——•): males (see text).

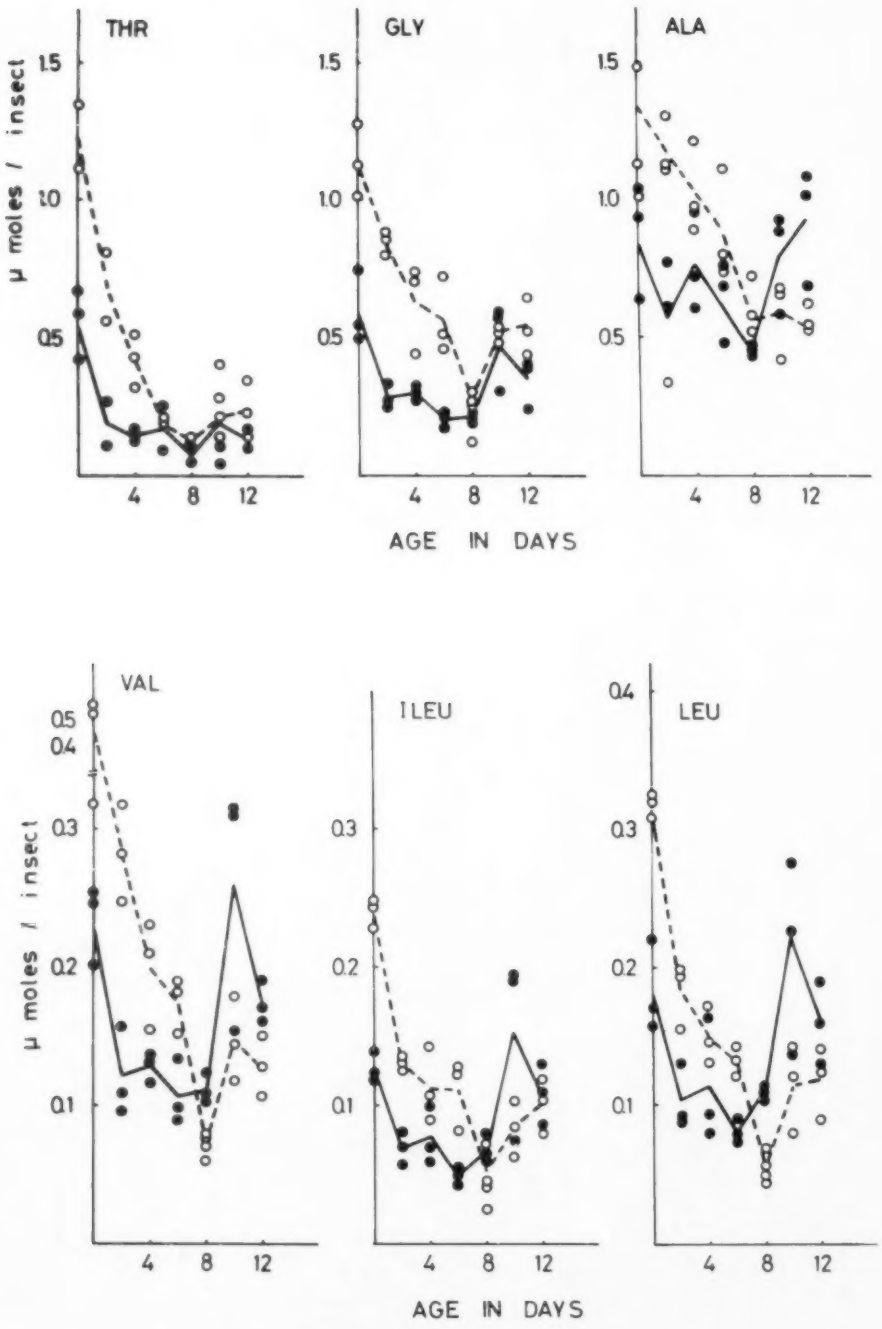

FIG. 3. Changes in the contents of free threonine, glycine, alanine, valine, isoleucine and leucine during adult lifetime of *Bombyx mori*. (o-------o): females, (•——•): males (see text).

Vol. 16 1981

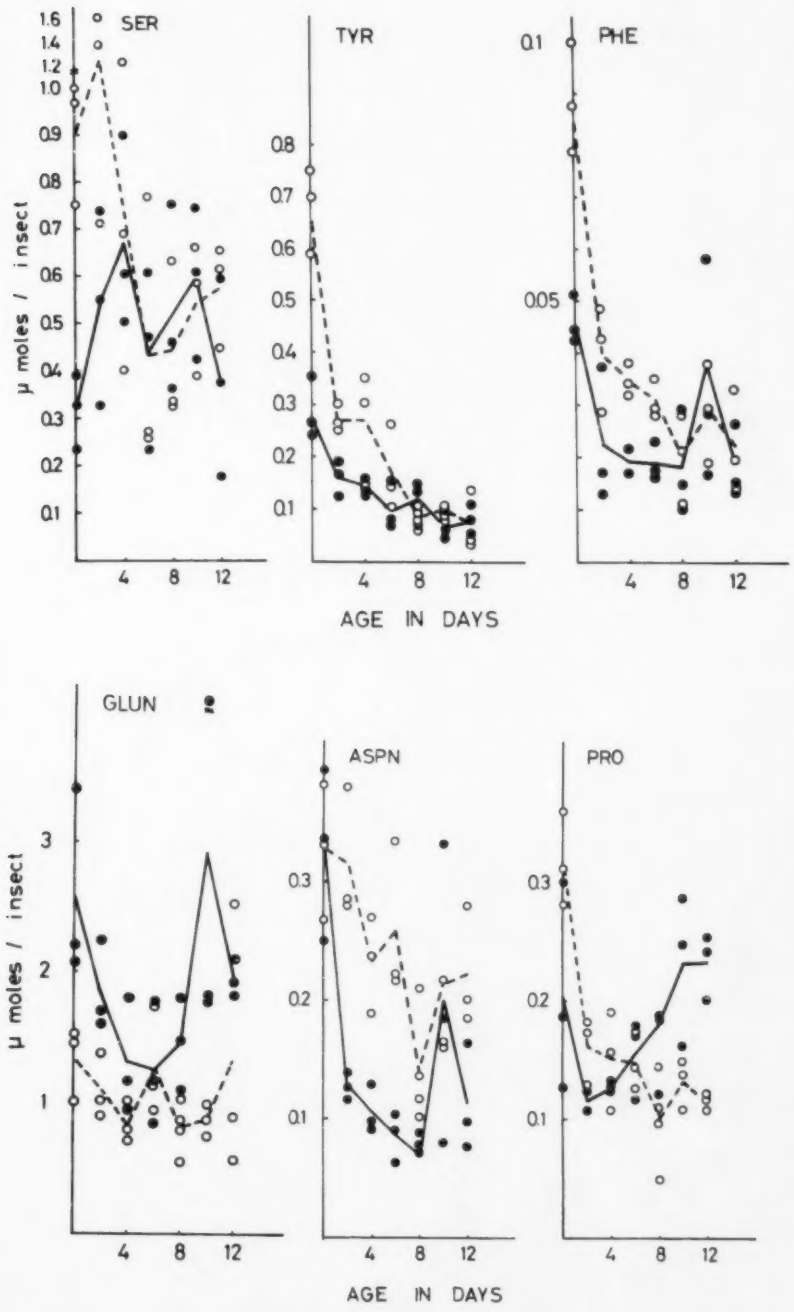

FIG. 4. Changes in the contents of free serine, tyrosine, phenylalanine, glutamine, asparagine and proline during adult lifetime of *Bombyx mori*. (o-------o): females (•————•): males (see text).

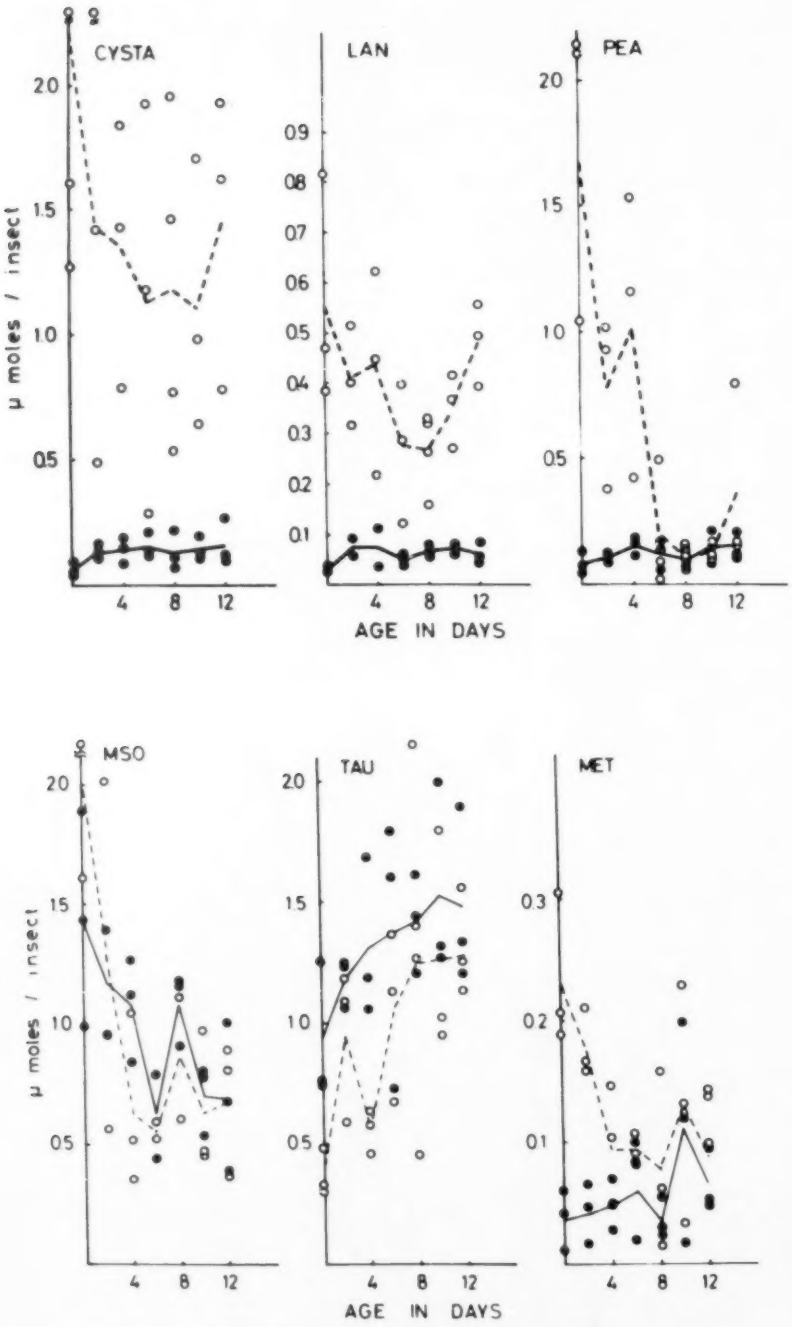

FIG. 5. Changes in the contents of free cystathionine, lanthionine, phosphoethanolamine, methionine sulfoxide, taurine and methionine during adult lifetime of *Bombyx mori*. (o-------o): females (•——•): males (see text).

ninhydrin-positive substances such as ammonia, methionine, histidine, asparagine, arginine, threonine, glycine, lysine, cystathionine, lanthionine and phosphoethanolamine were contained much more in females (Figs. 2–5). Especially, the level changes in the last three amino acids, taking place in early and middle age, were characteristic in females. The changes must suggest an important role of these amino acids on vitellogenesis and oviposition.

In early and middle ages the levels of valine, isoleucine, leucine alanine and proline were higher in females, while higher in males in the late stage (Figs. 3 and 4). Serine may belong to this group of amino acids (Fig. 4). Thus, one female silkmoth contained more total amino acids at high pool levels than one male moth (Fig. 1). Even when expressed per g body weight, the general tendencies mentioned above were not varied. A peak on the 6th day in females may be attributable to oviposition.

Other amino acids contained more in females, beside cystathionine, lanthionine and phosphoethanolamine, may act on oviposition as well as the related physiological phenomena (Fig. 5). However, even after finishing oviposition, the sexual differences were found as before. The sexual differences of the amino acid pools must, therefore, originate in differences of the sexes themselves, which are determined by a gene or genes on sex chromosomes.

Vol. 16 1981

β-alanine was detectable in females only just after emergence (162.8 n mole/insect). In males the amounts were about twice as much as in females (348.5 n mole/insect) on the day of emergence, but declined to 130.2 n mole/insect on the second day. Thereafter, β-alanine even in males became too slight to estimate in quantity. In *Drosophiia* it has been reported that the male fly contained about twice as much of this amino acid as the female (Kaplan *et al.*, 1958; Chen, 1972).

3. Aged changes in amounts of sulfur-containing amino acids

Cysteine and cystine were barely detectable during the whole imaginal life. Taurine increased continuously from emergence to day 12 (Fig. 5). Level changes of methionine, lanthionine and cystathionine belong to group M. Unlike other amino acids methionine sulfoxide formed a peak at day 8 (Fig. 5). In males the pool size of the amino acid is comparable with the summed value of the other sulfur-containing amino acids. In female moths cystathionine and methionine sulfoxide kept rather high levels. Whereas cysteine, cystine and methionine found in silkworm adults were little in amounts, metabolites such as taurine, cystathionine and methionine sulfoxide were rather large. The findings may suggest not only the active turnover of sulfur-containing amino acids but also the active synthesis as well as degradation of proteins in the insect.

4. Age-related changes in members in urea cycle

Since a functional change of excretion would be characterized as a physiological phenomenon in senescence, age changes in levels of members in urea cycle and some catabolites were investigated. Urea, one of main catabolic endproducts for secretion, and its forming system, the urea cycle, are broadly localized in animal kingdom. In the insect the existence of a complete urea cycle has, however, been doubtful (Szarkowska and Porembska, 1959; Pant, 1976). Another possibility, that urea is formed by breakdown of allantoic acid produced by uricolysis, is also unlikely in the insect (Cochran, 1975).

Neither allantoin nor allantoic acid were detected in silkworm adults. Amounts and

quantitative changes in members of the urea cycle were not considerable during the lifetime of silkmoths. Citrulline as well as argininosuccinate and urea were not found at all (Table 1). Ornithine was little in amounts. Changes of arginine levels were small compared with other amino acids (Fig. 2), especially in males. These results would suggest that the silkworm adult is probably lacking in a complete urea cycle, because of deficiency in ornithine-carbamoyl transferase. It may not be necessary for the moth to produce urea as an endproduct, which is easily soluble in water. Inokuchi *et al.* (1969) confirmed that labelled citrulline injected into *Bombyx* larva was metabolized to proline via arginine and ornithine. One cause of leveling-up of proline in the later stages of males (Fig. 4) may be its synthesis from orthinine. Proline would be utilized as an important substrate for movement of flight muscle as reported in some flies and other insects (Sacktor and Childress, 1967; Bursell, 1966; de Kort *et al.*, 1973).

DISCUSSION

When aged metabolic changes are studied in a closed-system insect like silkmoth, which lives on limited substances stored at larval stage, their analysis is generally easier compared with studies in an open system animal. Moreover, important features, which are hardly able to be detected in the open system, are sometimes emphasized in the closed system and appear in a more definite form. In the closed system insect excreting nothing, treatment of endproducts derived from catabolism of amino acids is a great problem in relation to aging. As in other insects (Porembska and Mochnacka, 1964) functional activity of the urea cycle was doubtful in the silkworm adult, as observed in the larva (Inokuchi *et al.*, 1969), since not only its important members such as argininosuccinate and citrulline but also urea were not detectable at all. Catabolic endproducts from nitrogen-containing compounds are obliged to take the varied forms of the other substances. Ammonia as well as taurine, increasing quantitatively in the late stage of the moth, must be two of these.

Among sulfur-containing amino acids methionine, cysteine and cystine were little in amounts. Cystathionine, lanthionine, methionine sulfoxide, and taurine, which were high in concentrations, changed their pool sizes markedly through adult life. However, the amounts of total sulfur-containing amino acids were always almost constant in spite of aging. Level decreasing after emergence of the silkmoth in cystathionine and lanthionine may suggest the conversion of these sulfur-containing amino acids into cystine, which accumulates in egg-shell protein in a large quantity (Kawasaki *et al.*, 1971). This may be one type of excretion of sulfur-containing amino acids, since their metabolisms are specifically active in the female adult. Some nitrogen-containing substances are excreted as endproducts with exuviae forming at each molt and metamorphosis (Horie *et al.*, 1978).

Quantitative increase of taurine, α-alanine and β-alanine and decrease of glycine, valine and lysine were found in aged male of *Drosophila melanogaster* (Chen, 1972). But unlike the fruit fly, in which most amino acids increase rapidly just after emergence and then decline, amino acids began to decline from emergence in silkmoths with a closed system (Table 2). β-Alanine was barely detectable only in the early stage of the moth. Therefore, the increase of taurine belongs to a common aging feature of both insect species. The decline of glycine, valine and lysine were also observed in silkworms, but there was rapid dynamic changes in the late stage.

In silkworm adults 36 free ninhydrin-positive substances were found, 35 of which were identified as amino acids and related substances. One unknown substance seemed to be a

Vol. 16 1981

TABLE 2. PARTITION (% TOTAL OF n mole/mg WEIGHT) OF FREE AMINO ACIDS IN INSECT ADULTS AT VARIOUS AGES†

Amino acid	Male Bombyx mori (A) – Drosophila melanogaster (B)*								Female Bombyx mori (C) – Aedes aegypti (D)**								
	A	B	A	B	A	B	A	B	C	D	C	D	C	D	C	D	
Age	0	0	2	3	8	20	12	30	2	9	4	16	8	30	12	45	days
Total (n mole/mg)	33.0	53.8	34.6	64.7	51.4	62.6	73.1	64.0	39.3	58.0	35.5	41.0	62.4	75.0	89.1	55.9	
Ser + Aspn + Asp + Glun + Glu	29.2	15.2	31.9	7.2	31.6	6.4	23.5	7.6	18.4	16.7	22.1	17.4	14.3	25.6	11.3	21.8	
Asp + Aspn	3.2	–	2.4	–	2.8	–	1.5	–	1.9	6.3	3.3	7.4	1.9	10.6	1.0	13.2	
Glu + Glun	23.3	–	22.4	–	23.6	–	19.2	–	9.3	6.0	13.4	5.2	8.4	10.2	7.8	5.6	
Pro	1.8	15.9	1.2	10.9	1.8	9.3	1.9	7.8	0.9	10.7	1.2	7.1	0.8	9.6	0.5	17.8	
Ser	2.7	–	7.1	–	5.2	–	2.8	–	7.2	4.5	5.4	4.7	4.0	4.9	2.5	3.1	
Thr	4.8	1.8	2.1	0.9	0.9	0.7	1.3	0.6	4.1	4.5	3.0	3.3	1.1	3.2	1.0	1.8	
Gly	5.3	4.6	3.2	3.6	2.2	3.6	2.8	3.1	4.8	3.7	4.5	3.8	2.3	3.1	2.3	3.0	
Ala	6.9	17.9	8.7	22.7	4.6	22.2	7.6	18.2	6.6	34.9	7.5	38.8	4.7	35.6	2.3	35.1	
Val	2.1	2.2	1.7	1.0	1.1	0.7	1.4	0.5	1.6	4.8	1.4	4.0	0.6	3.0	0.6	2.0	
Met	0.4	0.4	0.4	0.6	0.4	0.1	0.5	0.4	1.0	1.2	0.9	1.2	0.9	0.6	0.6	0.7	
Ileu	1.1	0.9	0.9	0.8	0.7	0.4	0.8	0.4	1.0	3.9	0.8	2.2	0.4	1.8	0.4	1.1	
Leu	1.6	1.2	1.4	0.9	1.1	0.6	1.2	0.3	1.0	4.9	1.1	4.2	0.5	3.3	0.5	0.9	
Tyr	2.6	1.3	1.8	0.1	1.2	0.3	0.5	0.3	1.5	0.7	1.9	1.9	0.7	1.0	0.3	1.3	
Phe	0.4	0.7	0.2	0.3	0.2	0.2	0.1	0.1	0.2	4.3	0.3	3.0	0.2	2.3	0.1	1.8	
Lys	1.1	4.8	0.9	1.5	0.6	1.1	0.7	1.3	1.7	2.2	1.1	2.4	1.1	1.5	0.7	2.2	
His	9.0	5.7	11.6	4.8	7.2	4.7	6.7	6.0	15.8	3.1	16.1	4.6	29.0	3.9	22.0	5.3	
Arg	4.3	4.7	3.8	3.9	2.7	3.9	2.7	4.1	6.7	4.4	5.5	6.0	2.2	5.8	2.2	5.3	

*P.S. Chen (1972);

**J.D. Stidham and J.N. Liles (1969), modified a little.

† Distributive percent (% total of n mole/mg body weight) of free amino acids in silkmoth (A and C) was compared with that in male fruit fly (B) and female mosquito (D) in various adult ages. Data from Chen (1972) (B), and Stidham and Liles (1969) (D) were cited after modified a little.

peptide. Among main 22 ninhydrin-positive substances, the pool sizes of amino acids belonging to group M changed very similarly to each other throughout the imaginal lifetime. The changes were divided into three stages, early, middle and late stages with advancing age. In the first stage just after emergence the amino acid pool drops rapidly to synthesize proteins necessary for postemergence maturation of the moth. The second stage, when the pools were rather stabilized, covers the middle age of the insect. Finally, the third stage is senescence: amino acid levels were dramatically changed in this late stage. These stages of silkworm adults were well in agreement with three phases in the whole lifetime identified by increase of mortality (Osanai, 1978). An individual having a high sensitivity, which cannot adapt to dynamic changes of metabolism occuring discontinuously, is bound to die. What rules over segments of life or "phases" must probably be drastic and dynamic change in amino acid-protein metabolism. Even in mammals, which undergo apparently continuous changes during growth, discontinuous changes in the body weight seem to make segments in the several stages of the lifetime (Morrison *et al.*, 1977). The segments may perhaps be induced by inner metabolic changes. So far several hypotheses have been posed on the aging of insects, principally on the basis of results obtained from various temperature treatments (Rockstein and Miquel, 1973), but there are few hypotheses sufficient for agreement among them. We must, therefore, reexamine experimental data accumulated hitherto with due regard to the segments, which reflect inner metabolic changes.

Quantitative changes of amino acid pools in adults of several insects with advanced age are presented in Table 2 (% total of n mole/mg body weight) and Table 3 (% total of n mole/insect). When distributive percent is expressed by "per mg insect," alanine is more in the male fruit fly and female mosquito than in silkworm moths. Histidine is, on the contrary, higher in silkmoths than in either of the dipteran insects. However, when expressed by "per insect," the partition of alanine in the moth agreed with other three species of insects. In both tables the silkworm moth showed also reciprocally different concentrations of glutamine (and glutamic acid) and proline from other insects, but the sum of both glutamine and proline takes generally a similar values in any insect. More glutamine or more proline in some insects must be linked to an equilibrium difference depending upon activities in enzymes such as proline oxidase, glutamate semihydrate dehydrogenase and proline-5-carboxylate reductase, which catalyse reactions among glutamine, glutamic acid and proline. More proline in flies must be due to their highly developed flight muscle, which utilizes this amino acid as a substrate for energy (Sacktor and Wormser-Shavit, 1966; Norden and Venturas, 1972).

With such a few exceptions, most partitions of amino acids in silkworm adult were surprisingly comparable with those in two species of open system insects, male of *Drosophila melanogaster* (Chen, 1972) and female of *Aedes aegypti* (Stidham and Liles, 1969) (Table 2) and also three species of dipteran insects (Table 3). In spite of different insect orders, different feeding habits and varied biochemical systems, amino acid-protein interactions may be very similar to each other among these insects. It is, therefore, very likely, that a similar homeostatic device for amino acid pools operates in holometabola. There may be a common principle for the regulation of amino acid pools in insects.

The dynamic changes in amino acid pools, the accumulation of free taurine and ammonia with advanced age caused by catabolism of proteins and the active metabolism of sulfur-containing amino acids suggest an active synthesis and turnover of proteins in silkworm moths. The individual difference of amino acid concentrations estimated in the

Vol. 16 1981

TABLE 3. PARTITION OF FREE AMINO ACIDS (% OF TOTAL AMOUNTS OF 14 FREE AMINO ACIDS) IN SEVERAL SPECIES OF INSECTS†

	Bombyx mori										
	Male				Female						
Amino acid	0	4	8	12	0	4	8	12	Drosophila* melanogaster	Drosophila* subobscula	Calliphora* erythrocephala
aspartic acid + asparagine	5.3	4.6	5.1	3.2	4.0	3.5	8.5	4.5	5.2	5.3	8.3
threonine	8.6	3.1	1.7	2.6	13.7	7.2	4.0	5.2	3.0	1.6	1.7
serine	5.0	14.3	11.7	7.4	10.1	13.4	13.7	12.7	8.8	5.5	8.4
glutamic acid + glutamine	42.0	42.5	52.8	47.0	20.7	31.5	36.1	41.5	25.9	27.7	30.4
proline	3.2	2.7	4.1	4.5	3.5	2.6	3.1	2.5	34.9	33.0	23.5
glycine	9.3	6.3	4.8	6.7	12.7	10.8	8.5	12.0	6.0	4.6	8.3
alanine	13.0	16.3	10.1	18.1	15.0	17.8	17.4	11.7	11.4	13.1	10.3
valine	3.7	2.7	2.5	3.4	5.4	3.4	2.2	2.8	1.2	3.4	1.1
isoleucine	2.0	1.6	1.5	2.1	2.7	1.9	1.6	2.2	0.6	1.6	2.7
leucine	2.8	2.4	2.5	3.2	3.6	2.6	1.7	2.6	1.0	2.3	3.8
tyrosine	4.5	3.1	2.7	1.5	7.6	4.6	2.6	1.6	1.4	1.5	0.7
phenylalanine	0.7	0.4	0.4	0.4	1.0	0.6	0.6	0.5	<0.5	0.5	0.6
total amino acid, n moles per insect	6,424	4,682	4,416	5,132	8,950	5,781	3,234	4,516	19	36	1,848

*J.I. Collett; *J. Insect Physiol.* (1976) **22**, 1251–1255.

†Distributive percent (% total of n mole/insect) of free amino acids in silkmoth in various stages was compared with three species of dipteran insects, of which data were cited from Collett (1976).

same imaginal stage was little deviated in spite of marked aging changes in pool sizes of most amino acids. Constancy of amino acid levels were also observed in worker pupae (Osanai, unpublished) and adult honeybees (McLeen, 1976).

It is likely that denaturation of glutaminyl and asparaginyl residues in protein resulted in a release of ammonia. Some deaminated proteins become easily decomposed (Flatmark, 1964; Robinson and Rudd, 1974). In the silkworm imago both free aspartic acid and glutamic acid are hardly detected. These amino acids were principally contained as a form of amide. However, when the moth is aged, a slight decrease of glutamine is accompanied by a simultaneous increase of glutamic acid, as reported in *D. melanogaster* (Robinson *et al.*, 1974). Such a reciprocal interaction was not found between asparagine and aspartic acid. Thus, the equilibrium between both systems of protein synthesis and degradation seem to be lost in senescence.

Of amino acids detected in silkworm imagines, most amino acids and the related substances demonstrated, more or less, quantitatively and sometimes even qualitatively, the sexual level changes throughout their lifetime. The changes were, moreover, observed even after maturation of ovary and subsequent oviposition. In the late stage the qualitative change differences between both sexes were found at the levels of proline, histidine, glutamine, cystathionine, lanthionine and alanine, whereas the quantitative ones were observed at the levels of isoleucine, leucine, taurine and ammonia. Since the moth has no sex hormones and, therefore, the physiological features are never influenced by the hormones like mammals, sexual differences of amino acid pools must probably be ruled directly by genes located in sex chromosomes. It is very interesting that for the total pool size of free amino acids, females are larger than males of silkmoths, having ZW-type sex chromosomes (Fig. 1), whereas males are larger in man, having XY-type chromosomes (Armstrong and Stave, 1973). Moreover, existence of sexual differences in changes in the levels of the majority of amino acids might strongly suggest a possibility, that some important factors to regulate amino acid-protein metabolism are located on sex chromosomes. It has been confirmed that a certain gene controls the incorporation of vitellogenin in haemolymph into ovary of *Bombyx mori* (Kawaguchi and Doira, 1973). Insect lives as well as other animal lives are thus maintained by finely complicated interactions and balances, regulated perhaps by development of gene actions, among synthesis and degradation of proteins, free amino acids and their catabolites. Death comes to a living thing, when one or more reactions among them are blocked or oppositely overstimulated. The longevity must be determined by the maintenance of the interaction balances. Old age in the silkmoth destroys these balances, and this was revealed by dynamic changes of amino acid pools and the accumulation of some endproducts of nitrogen-containing substances such as taurine and ammonia.

SUMMARY

Age-related changes in amino acid pool sizes were measured throughout the lifetime of silkworm adults representing a biochemically semi-closed system, since they do not feed after emergence. The moth contained 36 free amino acids and related substances and another unknown ninhydrin-positive substance, probably a peptide. The majority of amino acids among the 22 estimatable substances demonstrated very similar changes in pool: a rapid decline in the early stage after emergence, a stabilized pool in the middle stage and a rapid increase with a subsequent decrease in the late stage of old age. The ac-

cumulation of taurine and ammonia, which must be regarded as catabolites from amino acids and proteins, characterizes the senescence of the silkmoth. The sexual differences of changes in amino acid pools were observed quantitatively and qualitatively for several amino acids. It is very likely that genes located on the sex chromosomes regulate and control directly the amino acid pools. The stage-dependent stability of amino acid pools suggest actions of the homeostatic mechanisms in spite of active turnover of amino acids in this closed system. Analogies of both amino acid pool changes with advanced age and distributive percent of main amino acids between silkmoths and some dipteran insects suggest that amino acid-protein metabolism is controlled by a principle common to Insecta regardless of closed or open system. The urea cycle seem to be not functional in silkworm adults.

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Vol. 16 1981

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MOSAIC MAPPING OF FOCI ASSOCIATED WITH LONGEVITY IN THE NEUROLOGICAL MUTANTS *Hk*^{1} AND *Sh*^{5} OF *DROSOPHILA MELANOGASTER*

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Vol.
16
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INTRODUCTION

INSECTS POSSESS a number of characteristics which make them biologically and economically ideal for aging research: small size, high reproductive capacity, short lifespan, and an adult stage with virtually no cell division except for the gonads and, therefore, a relatively straightforward aging process. The fruit fly, *Drosophila melanogaster*, provides in addition to these attributes, a battery of mutants and manipulative techniques with which aging can be approached from a genetic point of view. One of these techniques, not heretofore applied to aging studies, is mosaic fate mapping. Mosaic flies are composites of two clones of cells; in the present case one clone carries the normal genetic determinants of longevity, while the other carries, along with markers for external cuticular structures, a mutant gene which shortens the lifespan. By observing the lifespans of these mosaics it should be possible to define an aging focus on the fate map of the blastula and to infer the corresponding adult tissue most closely associated with this aging process. A curve-fitting method was developed and applied to the data obtained by this technique, for the two neurological mutants *Hyperkinetic*^{1} (*Hk*^{1}) and *Shaker*^{5} (*Sh*^{5}), which have a reduced lifespan correlated with an elevated physical activity level and rate of oxygen consumption (Trout and Kaplan, 1970). The focus of this effect could therefore be a fundamental aging center controlling activity level or metabolic rate. For both mutants, the analysis suggested that this focus is located in the nervous system, mapping to the anterior thoracic ganglion. At the same time, there is another focus, responsible for unusually early death in mosaics, mapping laterally near the developmental region for the wing. This early death focus has nothing to do with the mutations and is probably the result of detrimental juxtapositions of male tissue in the wing region, and female tissue anteriorly. This curve-fitting method is an exploratory

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model for future mosaic mapping in studies of the genetics of aging, and may also lend itself to the analysis of other quantitative and multi-focus characters.

METHODS AND PROCEDURE

Subjects

The mutants *Hk*^{1} and *Sh*^{5} previously described by Trout and Kaplan (1968), and Kaplan and Trout (1969), shake their legs vigorously when etherized and so, along with other mutants of this type, are called "shakers." Both mutations are on the X-chromosome which for the purposes of these experiments was also marked with yellow body and white eye mutations (*y*,*w*) in order to monitor the phenotype of the adult cuticle. To provide the same genetic background in the two mutant stocks and *y w* controls they were repeatedly backcrossed to the same standard wild type, *Canton-S*, stock in which the mutants arose.

Hk^1 and *Sh*^{5} were chosen as aging mutants because of their effect upon longevity (Trout and Kaplan, 1970). The mortality curves of the two mutants appear to be normal in shape, but shifted to the left of *Canton-S*, and the Gompertzian mortality rate (the number of flies dying on a given day per flies alive at the beginning of that day) increases logarithmically with age, as with normal aging, but at a faster rate. The reduction in longevity in these mutants is correlated not with a morphologic abnormality but with an elevated activity level and rate of oxygen consumption. It is possible, therefore, that the effect on lifespan is through general metabolic rate and that these mutants would lend themselves to studies of aging in which the genetic technique of mosaicism may be exploited.

Mosaics

The gynandromorphs (mosaics) have been generated by use of a mating scheme designed to produce female zygotes whose sex chromosome complement is made up of a normal rod-X, and an unstable ring-X designated *In(1)w*^{vC} by Hinton (1954, 1955). The rod-X carries the mutant gene of interest along with cuticular and eye color markers while the ring-X carries their normal dominant alleles. It is generally regarded that the ring-X is lost, with predictable frequency, in one of the daughter nuclei during the first or second cleavage division (Hall *et al.*, 1976). This situation gives rise to one daughter nucleus containing only the rod-X chromosome which then, through succeeding divisions develops into a clone of male (mutant) cells which, in the appropriate tissues, express the marker gene phenotype and the mutant character being studied. The other daughter nucleus carries both the rod and the ring-X and gives rise to female tissue phenotypically wild-type. The respective cuticular areas, male or female, may therefore be readily distinguished and mapped (Ikeda and Kaplan, 1970; Hotta and Benzer, 1970).

Because the spindle orientation of the first cleavage division is not constant, the line establishing the male-female demarcation may vary in position resulting in a variety of mosaic types. Sturtevant (1929) used mosaics in exploiting this property of *Drosophila* embryology in order to establish embryonic relationships between adult structures. He recognized that the probability that two given structures may be of different genotype, in a population of mosaics, is determined by the developmental distance between them. Using these probabilities one can construct a two-dimensional representation of the surface of the blastoderm (Fig. 1) showing the relative positions of the presumed progenitors of adult and larval structures. These representations may, in turn, be superimposed upon the *Drosophila* fate map constructed by Poulson (1950) which indicates the regions destined to give rise to specific imaginal tissues and organs.

External markers may be identified easily but the genotype of internal tissues cannot be determined by inspection. Hotta and Benzer extended the study of mosaics into the field of behavior genetics by devising a method for determining which internal tissues have been made defective by behavioral mutants (Hotta and Benzer, 1972, 1973). By so doing they were able to define particular regions of the blastoderm, developmental foci, which were assumed to give rise to the adult organ or structure responsible for the observed behavior. This technique makes it possible to place the primary focus of the behavioral defect squarely into the primordial site in the nervous or muscle system, for example, rather than into some secondary organ, such as the leg, which exhibits the mutant behavior. In addition, it is possible to determine for a whole-body behavior, which involves a bilateral pair of foci, whether both foci must be mutant for the mosaic to be mutant (termed a submissive focus), or if only one mutant focus is sufficient (a domineering focus). In this way, analyses of behaviors such as phototaxis, motor system defects, circadian rhythms and courtship have been analyzed (Ikeda and Kaplan, 1970; Hotta and Benzer, 1970, 1972, 1976; Konopka and Wells, 1979; Hall, 1977). In the experiments herein reported we have tried to use aging as Hotta and Benzer have used behavior in order to see whether a developmental focus for the control of aging may be uncovered.

The mosaics in our study appeared in the F_1 generation of crosses of females of the *In(1)w*^{vC} stock to males that were either *y w Hk*^{1}, *y w Sh*^{5} or in the case of the control mosaics, *y w*. Although the unstable ring-X chromosomes in use today are descendants of the one originally described by Hinton we used two stocks obtained

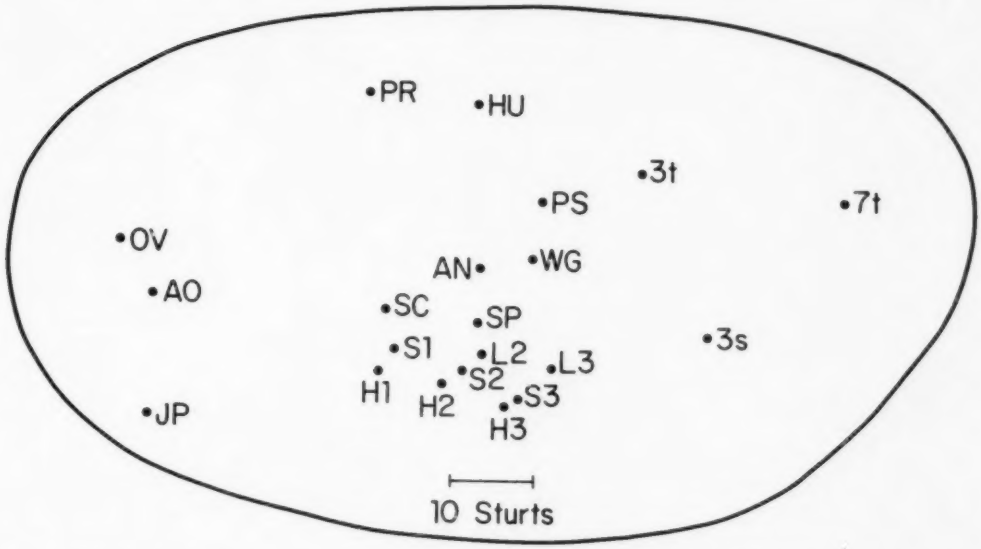

FIG. 1. Side view of the blastoderm, with the head to the left, showing the relative positions of selected cuticular landmarks, *Hk*[1] and *Sh*[5] shaking, and *Hk*[1] jumping behavior. Abbreviations: AO, Anterior Orbital Bristle; OV, Outer Vertical Bristle; PR, Proboscis; HU, Outer Humeral Bristle; PS, Posterior Scutellar Bristle; WG, Margin of Wing; AN, Anterior Notopleural Bristle; SP, Anterior Sternopleural Bristle; SC, Sex Comb; L2, Preapical Bristle of Midleg; L3, Preapical Bristle of Hindleg; H1–H3, HK[1] shaking; S1–S3, Sh[5] Shaking; 3s, Third Sternite; 3t, 7t, Third and Seventh Tergites; JP, *Hk*[1] Jumping.

from different sources: the chromosome here designated as "A" ring which was used for the *Hk*[1] experiment and its controls (A controls), had been balanced over the *delta-49* rod-X chromosome inversion, *In(1)d1-49*; whereas the "B" ring, used for the *Sh*[5] experiment and the B controls had been carried in a stock with a normally ordered rod-X-chromosome carrying the recessive markers *y w* and *split (spl)*. Because the male tissue in mosaics carries no Y-chromosome, males without a Y (XO) were used as non-mosaic controls.

At least 300 mosaic flies from each of the four crosses were collected and etherized as lightly as possible within a week of eclosion in order to record on fly diagrams (Fig. 2) the distribution of *y w* and normal external cuticle, and the presence of shaking legs. In addition, *Hk*[1] mosaics were scored for mutant jumping behavior when awake (Kaplan and Trout, 1974). The mosaics were kept individually in numbered 28 ml shell vials with 6 ml of a standard *Drosophila* food (Lewis, 1960). Vials were kept horizontally in order to lessen the likelihood of flies becoming stuck in the food, and were maintained at 25 ± 0.5°C at approximately 50% relative humidity. A fresh food vial was provided every other day.

The identification number, age in days at death, and the phenotype of 14 bilateral cuticular landmarks (plus leg shaking in the *Hk*[1] and *Sh*[5] mosaics, and jumping for *Hk*[1]) were recorded for analysis on a PDP-11/34 computer. These landmarks (Fig. 1) were selected in order to cover the surface of the blastoderm as well as possible, with the most reliably scored sites. To facilitate analysis, the phenotypes of specific individual bristles or restricted areas of the cuticle were used in order to be able to score each landmark as male or female, eliminating a mixed category. The least-squares computer mapping technique developed by Flanagan (1976) was used to estimate the relative positions of landmarks shown in Figure 1, which is based on the data from *Hk*[1] mosaics; *Sh*[5] and the controls had similar maps. The Gompertz plot parameters in Table 1 were calculated by a computer program which smoothed the curves of deaths per day (as in Fig. 3) and used only the linear part of the Gompertz curve.

RESULTS

Localization of shaking and jumping behavior

For both *Hk*[1] and *Sh*[5] the shaking of each leg maps as a single landmark, distinct from the leg cuticle foci. The *Hk*[1] shaking foci (H1-H3 in Fig. 1) map 8–9 sturts ventral to the

Vol. 16 1981

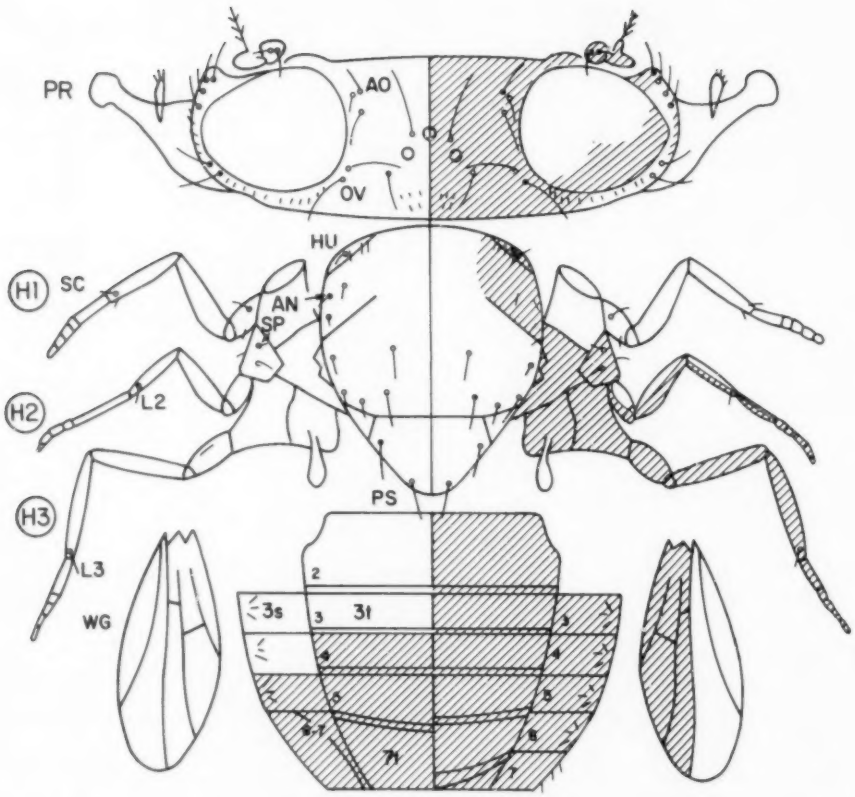

FIG. 2. Representative mosaic score sheet showing the imaginal cuticular landmarks used in the data analysis. Female tissue shaded, *y w Hk*^{1} male tissue unshaded. In this case all three left legs (H1-H3) were observed to shake as indicated by the circles.

respective leg foci; this agrees with data previously obtained by Hotta and Benzer (1972) and in this laboratory. The *Sh*^{5} foci (S1-S3), added to the *Hk*^{1} map, (Fig. 1) appear to map more closely to the leg, in this experiment 5, 4 and 7 sturts, respectively, ventral to the three leg foci. In agreement, Ikeda and Kaplan (1970) by means of physiological techniques have shown that the *Hk*^{1} shaking is controlled by neurons in the pro- meso- and metathoracic motor regions of the thoracic ganglion and are separate from the leg cuticle; it is presumed that this is true for *Sh*^{5} as well.

In addition to shaking its legs while etherized, *Hk*^{1} is also abnormally sensitive to movement or a strong flashing light while awake, "jumping" uncontrollably when stimulated. All of the *Hk*^{1} mosaics were tested for this trait by placing them individually in an empty 28 ml shell vial and waving a hand over them. This behavior, when analyzed by the method of Hotta and Benzer (1972) or the focusing method of Flanagan (1976) is domineering (exhibits the mutant behavior even if the focus on only one side of the fly is mutant) and maps about 6 sturts from the ventral midline in the head, distinct from the eye or head cuticle, and probably in the cephalic ganglion. The ventral location agrees with results from other *Hk*^{1} mapping experiments (Kaplan, 1976).

TABLE 1. AVERAGE LIFESPANS OF MOSAIC AND CONTROL GROUPS

Stock	Flies	Lifespan, days after eclosion ±S.E.	T_{50} days	Gompertz plot, log mortality rate = a + b days ± S.E. a	b	S.E.
Mosaics						
y w Hk^1/A ring (Hk^1 mosaics)	292	74.1 ± 1.3	76	−3.07	0.021	± 0.04
y w/A ring (A-control mosaics)	281	78.5 1.6	82	−3.14	0.020	0.05
y w Sh^5/B ring (Sh^5 mosaics)	316	75.1 1.3	78	−3.13	0.021	0.07
y w/B ring (B-control mosaics)	306	79.8 1.3	83	−3.51	0.024	0.06
Non-mosaics						
y w males (XO)	277	94.5 1.0	96	−4.65	0.034	0.14
y w/A ring females	277	91.0 1.4	95	−3.80	0.025	0.11
y w Hk^1 males (XO)	309	73.3 0.8	74	−4.15	0.037	0.13
y w Hk^1/A ring females	289	88.4 1.4	94	−3.70	0.024	0.05
y w Sh^5 males (XO)	277	77.3 0.9	77	−3.71	0.030	0.07
y w Sh^5/B ring females	310	88.7 1.2	94	−3.69	0.025	0.09

Vol. 16 1981

Estimation of aging curve parameters

The percent deaths per day for the mosaic and control experiments are shown in Fig. 3, and the mean lifespans and Gompertz curve parameters in Table 1. As expected, the mutant non-mosaic males died significantly earlier than the normal (*y w*) males; the reduction in lifespan was 22% for Hk^1 and 18% for Sh^5. This is less than the 48% reduction for

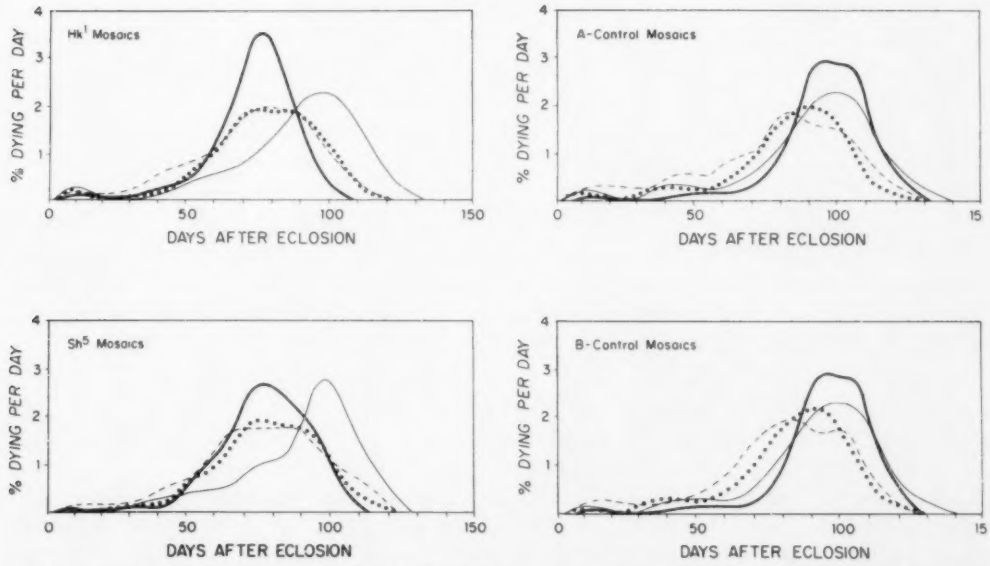

FIG. 3. Percent deaths with age for mosaics (dashed lines) and their non-mosaic male (heavy lines) and female (light lines) controls. The dotted lines are best fits to the mosaic curves, obtained by combining and manipulating the non-mosaic curves.

homozygous Hk^1 and Sh^5 females reported previously (Trout and Kaplan, 1970); both the shakers and controls lived longer in the present experiments, when kept one per vial, than previously when 50 per bottle. This difference may be the result of less hazardous conditions for senile flies in a vial kept horizontally, where there is less chance of sticking to the food, and because flies kept individually, especially shakers, are less active than in groups (Kaplan and Trout, 1968). Flies kept singly also have a lower metabolic rate per fly than in groups of 20 (unpublished data). According to Rubner's hypothesis (1908) single flies should, therefore, age more slowly and live longer than flies maintained in groups.

Heterozygous Sh^5 and Hk^1 females had only a slight reduction in lifespan; for studies of aging, both Hk^1 and Sh^5 can therefore be considered recessive even though this is not true of leg shaking, which is recessive in Hk^1 and partially dominant in Sh^5 (Trout and Kaplan, 1973). The non-mosaic *y w* males lived slightly longer than the non-mosaic *y w*/ring females. Therefore, *y*, *w* and maleness as such would be expected to have no deleterious effect upon lifespan. However, both A and B control mosaic groups died about 15% earlier than these non-mosaic controls, significant at the 1% level. This aspect will be discussed later because it appears that mosaicism itself has a deleterious effect upon lifespan.

As expected, the Hk^1 and Sh^5 mosaics died earlier than the control mosaics. The reduction is about 6% in both cases and is significant at the 5% level, but the actual mutant effect must be greater than this because only a portion of the Hk^1 and Sh^5 mosaics should be short-lived, having mutant tissue in the critical places to cause life-shortening. It is not possible in these experiments to tell which mosaic individuals are long- or short-lived, because the mosaic age curves (Fig. 3) did not resolve themselves into two distinct categories. Attempts to resolve this problem by using the mean lifespans of the mosaics and controls, and of particular mosaic types (as in Table 2), were not satisfactory, probably because, as will be shown, more than one aging focus happens to be involved. Much better results were obtained by using the aging curves themselves, and assuming that each mosaic curve is the summation of two aging curves, those of the appropriate short-lived (male) and long-lived (female) non-mosaic control types.

An empirical curve-fitting computer method was developed to manipulate these non-mosaic control curves, sum them, and compare the result with the observed mosaic aging curves, to estimate the proportions of long- and short-lived mosaics which best fit the data. This method made it possible to treat each mosaic experiment as its own control, eliminating the need for detailed comparisons of two different collections of mosaics, the mutants and controls, each subject to variation. This also allowed the leg shaking phenotype to be used in the analysis of the mutant mosaics, even though leg shaking cannot be scored in the control mosaics. In addition, because the aging curves were used, rather than only their mean lifespans, it was possible to detect and map a second lifespan focus, associated with mosaicism itself.

The curve-fitting method assumes that the mosaic aging curves in Fig. 3 are derived in some way from the appropriate male and female non-mosaic control curves. For example, it should be possible to duplicate the Hk^1 mosaic curve by combining the non-mosaic Hk^1 male curve (representing the short-lived mosaic types) and the heterozygous Hk^1 female curve (representing those mosaics with normal lifespan). These two curves must first be reduced in area according to the contribution of each, so as to add up to the original 100%. The resultant calculated curve must also be shifted to the left a certain amount to

TABLE 2. AVERAGE LIFESPANS AND CALCULATED CURVE-FITTING PARAMETERS P, D AND M, FOR MOSAICS WITH A GIVEN LANDMARK MALE ON BOTH SIDES (MM), ONE SIDE (MF) OR NEITHER SIDE (FF).*

Hk[1] Mosaics

	Mosaics			Lifespans			% Short-lived (P)			% Early deaths (D)			% Mosaic effect (M)		
	MM	MF	FF	MM	MF	FF	MM	MF	FF	MM	MF	FF	MM	MF	FF
AO	79	94	119	73	72	77	48	27	36	18	7	8	5	13	3
OV	81	92	119	74	73	76	33	34	23	12	6	9	9	13	6
PR	90	98	104	71	77	75	47	23	29	16	6	11	6	10	8
HU	89	114	89	72	75	76	63	29	24	16	9	11	3	8	8
PS	66	168	58	68	75	80	31	50	6	22	6	19	14	3	8
WG	61	168	63	67	75	81	49	52	0	31	5	10	12	3	9
AN	71	158	63	68	76	79	57	52	4	16	7	16	12	1	8
SP	86	140	66	67	76	82	57	69	0	17	9	9	12	-2	8
SC	92	136	64	71	75	78	61	25	0	11	8	11	8	7	12
L2	84	138	70	68	76	80	54	34	0	14	7	6	11	6	11
L3	84	137	71	68	75	80	53	52	0	22	4	9	13	3	10
S1	95	117	80	71	78	74	58	25	5	12	5	17	9	6	12
S2	82	128	82	69	74	80	61	21	0	16	6	6	11	10	11
S3	104	111	77	70	76	79	54	49	0	15	8	8	11	1	12
3s	63	148	81	70	76	76	56	33	20	29	6	9	7	6	10
3t	73	129	90	69	75	78	2	41	24	23	9	5	17	6	8
7t	72	108	112	70	77	75	21	31	30	22	7	8	13	7	9

Sh[5] Mosaics

	Mosaics			Lifespans			% Short-lived (P)			% Early deaths (D)			% Mosaic effect (M)		
	MM	MF	FF	MM	MF	FF	MM	MF	FF	MM	MF	FF	MM	MF	FF
AO	89	81	146	75	76	75	61	81	53	11	14	9	5	-1	5
OV	81	84	151	74	76	76	62	66	67	15	13	8	5	2	2
PR	76	96	144	67	80	77	53	70	60	20	9	7	12	-1	2
HU	84	109	123	68	77	79	32	69	41	17	6	8	15	5	2
PS	66	156	94	68	77	79	38	66	24	16	4	10	17	5	3
WG	54	162	100	67	75	80	88	69	26	27	5	7	9	5	2
AN	61	160	95	67	76	80	58	75	31	18	6	9	13	2	2
SP	69	148	99	67	76	81	76	81	19	23	6	7	11	2	4
SC	72	145	99	68	75	81	76	86	21	25	6	8	10	2	2
L2	60	145	111	66	75	81	87	77	26	25	8	5	10	2	4
L3	71	141	104	66	75	82	86	68	26	22	5	6	10	5	2
S1	76	134	106	68	75	82	74	76	23	24	4	5	10	6	2
S2	67	135	114	67	75	80	76	80	29	23	7	5	11	2	4
S3	96	120	100	69	77	79	79	61	26	17	4	8	8	7	3
3s	63	126	127	68	71	83	81	56	32	19	8	2	9	11	2
3t	61	109	146	66	72	82	42	77	33	25	7	4	16	8	2
7t	72	102	142	65	72	83	73	33	43	41	5	2	8	15	2

*Abbreviations as in Fig. 1.

Vol. 16 1981

TABLE 2. (continued)

A-Control mosaics

	Mosaics			Lifespans			% Early deaths (D)			% Mosaic effect (M)		
	MM	MF	FF	MM	MF	FF	MM	MF	FF	MM	MF	FF
AO	78	77	126	72	82	80	31	9	12	14	7	7
OV	81	67	133	71	82	81	30	10	11	15	7	5
PR	102	85	94	74	80	82	19	17	14	11	7	7
HU	110	91	80	73	79	85	21	12	15	9	12	2
PS	76	157	48	74	79	81	31	8	24	9	9	7
WG	62	173	46	71	80	81	48	7	28	11	9	4
AN	62	165	54	71	80	81	47	7	27	11	9	2
SP	73	150	58	74	79	81	29	10	26	13	7	2
SC	70	137	74	74	78	82	36	8	19	9	12	2
L2	63	148	70	72	78	84	47	10	14	14	9	2
L3	73	142	66	73	78	85	28	13	9	15	5	4
S1	–	–	–	–	–	–	–	–	–	–	–	–
S2	–	–	–	–	–	–	–	–	–	–	–	–
S3	–	–	–	–	–	–	–	–	–	–	–	–
3s	68	146	67	75	76	87	28	11	10	12	12	1
3t	72	139	70	70	77	88	42	9	6	7	14	2
7t	79	107	95	70	79	85	31	10	11	12	13	2

B-Control mosaics

	Mosaics			Lifespans			% Early deaths (D)			% Mosaic effect (M)		
	MM	MF	FF	MM	MF	FF	MM	MF	FF	MM	MF	FF
AO	88	63	155	89	78	76	3	21	7	3	12	16
OV	88	60	158	86	82	76	4	23	6	5	9	16
PR	89	81	136	83	81	77	13	7	12	6	14	9
HU	77	122	107	80	79	81	14	10	12	9	9	7
PS	62	150	94	80	79	80	19	8	13	9	9	9
WG	50	168	88	80	79	82	22	8	10	9	9	9
AN	55	165	86	80	79	81	22	7	14	9	9	7
SP	49	160	97	84	78	81	19	7	11	5	11	9
SC	69	144	93	86	77	79	5	9	13	6	14	9
L2	45	150	111	82	78	82	25	8	9	5	11	7
L3	64	146	96	82	78	81	14	9	11	9	9	9
S1	–	–	–	–	–	–	–	–	–	–	–	–
S2	–	–	–	–	–	–	–	–	–	–	–	–
S3	–	–	–	–	–	–	–	–	–	–	–	–
3s	45	140	121	75	79	82	25	7	8	16	11	7
3t	61	130	115	80	77	83	7	10	10	14	11	7
7t	75	106	125	77	76	84	6	16	7	17	9	5

simulate the observed reduction in lifespan of mosaics, relative to the non-mosaic controls. For this purpose, a FORTRAN program was developed to calculate the percent of flies dying per day in each group and smooth the resulting curves in order to remove small fluctuations; these are the curves shown in Fig. 3. The program then took a percentage (P)

of the appropriate male non-mosaic control group (such as Hk^1 males) by multiplying all the daily percent deaths by P%; took the rest (100%-P) from the female control group; and added the resulting curves together. This calculated curve was compared with the Hk^1 mosaic curve by determining the sum of the squares of the differences between them, point-by-point. The program iterated through the range of values of P, from 0 to 100, to determine the value which gave the best fit to the mosaic curve and, therefore, the best estimate of the percentage of short-lived mosaics in the experiment.

Because even the control mosaics had a shorter lifespan, by about 20%, than the non-mosaics, a second factor, M, was iterated through at the same time to shift each point in the calculated curve to the left by percentages ranging from 0 to 20. The value of M (in conjunction with P) giving the best fit would then indicate the effect upon lifespan of mosaicism itself, irrespective of the presence or absence of mutant tissue.

The resultant best-fit calculated curves generated by P and M still left a shoulder of the mosaic curves unaccounted for in all four groups. This shoulder, between approximately 20 and 70 days of age, appears to represent a class of mosaics which died unexpectedly early for reasons having nothing to do with the shaker mutations. Therefore, to fit the mosaic curves properly, the calculated curves had to be reduced in area by a factor ranging from 0 to 20% to subtract the percentage of early deaths comprising this shoulder. Once the best fit was obtained, the percentage of early death, D, was estimated by subtracting the calculated curve from the mosaic curve wherever the shoulder was present, up to an arbitrary limit of 70 days after eclosion.

Vol. 16 1981

The best fit curves resulting from this analysis are shown in Fig. 3 (dotted lines). The estimate of P in the Hk^1 mosaic experiment is 29% short-lived types; since this is less than 50%, the Hk^1 mutant effect is probably submissive. In contrast the life-shortening effect of Sh^5 is domineering since, in its case, P is 69%. The percentage of early deaths, D, is 9% for Hk^1, 15% for the A controls, 12% for Sh^5 and 11% for the B controls; and the estimated reduction in lifespan, M, due to mosaicism (after accounting for D) is 2% for the B controls and 9% for the others. Such estimates are clearly more reliable when the control and mosaic curves are smooth and regular, and result in a good fit. Since the Hk^1 curves are the most ideal in this respect, the best estimate for both D and M, for mosaics in general, is about 9%.

The curve-fitting method used above for the mosaic experiment as a whole may also be used to estimate identical parameters for any sub-set of mosaics, such as those with both AO bristles male. From the percentage, P, of short-lived mosaics of each subclass, the number of such mosaics can be calculated and used in the equations of Hotta and Benzer (1972) to estimate the distance between each landmark and the lifespan focus. The values of P, D and M for each landmark obtained by this method are shown in Table 2, and the calculated distances to the mutant focus are shown in Fig. 4, arranged on a blastoderm map in the same relative positions as the landmarks in Fig. 1, with the head to the left. Because the values for distance do not point to a simple focus for the mutant effect, the "contour mapping" technique introduced by Hotta and Benzer (1976) has been used. Both Hk^1 and Sh^5 mutant foci appear to map to the anterior ventral region of the blastoderm, possibly to the anterior part of the thoracic ganglion. In agreement with calculations of P discussed earlier, the Hk^1 focus is submissive, and the Sh^5 focus is domineering. The distance between mutant foci, from one side to the other, is about 45 sturts for Hk^1 and 30 for Sh^5.

From the proportion of early deaths, D, the number of early deaths can be obtained

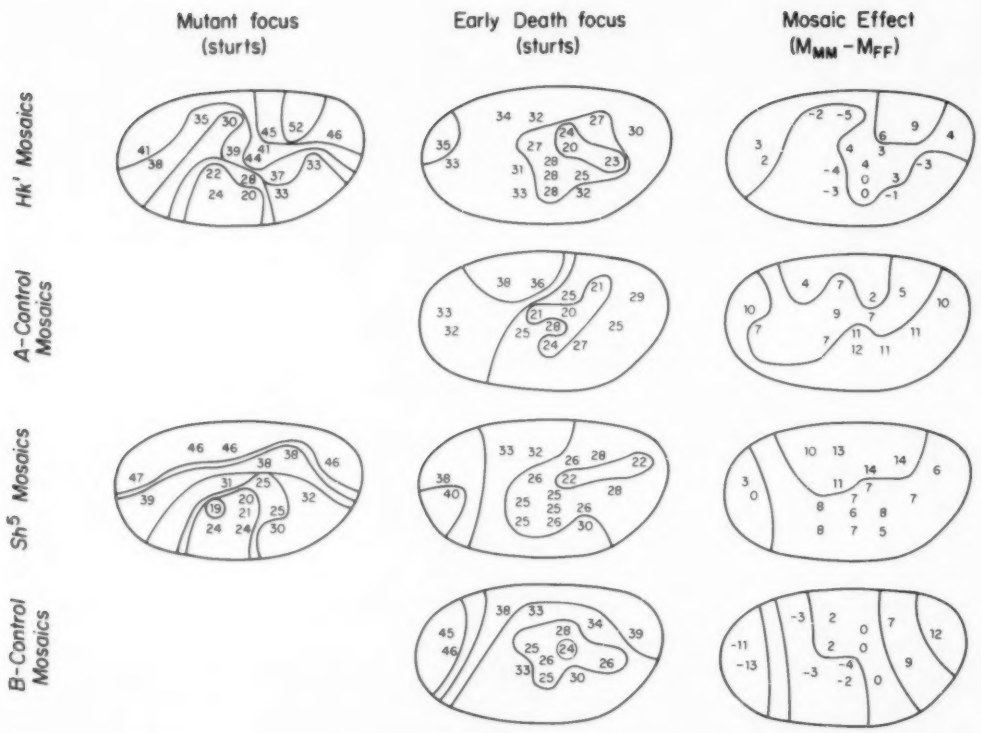

FIG. 4. Contour maps of distances in Sturts from each landmark to; (1) the focus for Hk^1 and Sh^5 life-shortening; (2) to the focus for early mosaic death; and maps of the deleterious effect of male relative to female tissue on lifespan, after accounting for foci (1) and (2).

and used in the Hotta and Benzer equations to calculate the distances to an "early death" focus; these are shown in Fig. 4. All four experiments indicate a similar submissive focus centered in the developmental region of the wing. The distances between left and right early death foci are 76 for Hk^1; 61 for the *A*-controls; 73 for Sh^5 and 76 for the *B* controls.

The third factor, *M*, is the percent reduction in lifespan of each mosaic category after accounting for the mutant and early death effects. It is not possible to calculate sturt distances as with *P* and *D*, but the relative effect of male tissue in each region defined by the chosen landmarks can be estimated by subtracting the value of *M* when both left and right landmarks are female, from that when male (Fig. 4).

DISCUSSION

The life-shortening effects of both Hk^1 and Sh^5 appear to map to the same anterior ventral region of the blastoderm, even though the analysis involved two different shaker mutations, two different unstable ring-X stocks, and two different sets of non-mosaic controls. This provides some reassurance that the analysis by the curve-fitting technique is a step in the right direction and suggests that the two mutations may affect lifespan in the same way, perhaps through neuronal activity in the anterior part of the thoracic ganglion. However, the effect is submissive for Hk^1 and domineering for Sh^5, that is, the lifespan

focus would have to be mutant on both sides of an Hk^1 mosaic to produce life-shortening, but need be mutant on only one side in Sh^5. A computer model taking the Hk^1 and Sh^5 mosaic landmark data (but not the lifespans) and using leg shaking to simulate a hypothetical aging focus in the thoracic ganglion, generated contour maps roughly similar to those observed for the mutant effect, by assuming either a single paired aging focus in the prothoracic ganglion (for example, designating an Hk^1 mosaic as "short-lived" if both forelegs were shaking) or a multiple site in the pro- and mesothoracic ganglia ("short-lived" if more than half of the fore- and midlegs were shaking). A model involving all six legs or only the mid-and hind legs, did not generate contour maps similar to those observed. In addition, there is little correlation between mutant life-shortening and Hk^1 jumping, which maps to the brain. Therefore the mutant focus appears to lie in the anterior part of the ventral thorax.

There is no compelling evidence at this point to distinguish between a multiple site focus and a simple one for either Hk^1 or Sh^5. The percentage of short-lived Hk^1 mosaics determined by curve-fitting, 29%, is consistent with either the model of a simple submissive focus (33% of the Hk^1 mosaics had both forelegs shaking) or a multiple submissive focus (the same percent had more than half of the fore- and midlegs shaking). The estimated percentage of short-lived Sh^5 mosaics, 69%, is consistent with either the model of a simple domineering focus (66% of the Sh^5 mosaics had at least one foreleg shaking) or a multiple domineering focus (69% had one or more fore- or mid-legs shaking). The 30–45 sturts between left and right lifespan foci agrees with the 33–47 sturts predicted by the model (the left and right shaking foci are 38–44 sturts apart), but the landmark-to-focus distances are 10–20 sturts longer by curve-fitting than expected by the model. Even in the multiple-site models the distance from the simulated "mutant" focus to leg shaking is not greater than 5 sturts, yet in our experiment, all of the landmarks are at least 20 sturts away. This could mean either that the aging focus is some distance ventral to the shaking foci, or that the lifespan focus is in fact somewhere in the ganglion, and the landmark-to-focus distances are all artificially large.

The "early death" contour maps indicate that there is a deleterious effect of male tissue in all four experiments, even in control mosaics without shaker mutations, focusing roughly in the developmental area of the wing. The left and right foci are 61–76 sturts apart, which is reasonably close to the observed left-to-right wing foci distances of 52–62 sturts. However, as with the mutant lifespan foci, the landmark-to-focus distances are about 20 sturts larger than expected in the region of the focus, but here there is no empty ventral region on the fate map which might absorb the extra distance. Therefore it is probable that the contour-map distances to both the early death and mutant foci are artificially high, perhaps because of the statistical nature of the aging phenotype and its analysis.

Although the mutant focus can be ascribed to the presence of abnormal Hk^1 or Sh^5 tissue in the ganglion, the early death focus, found in both mutant and control mosaics, may be associated with mosaicism itself, and exert its effect not merely through the presence of male tissue in the developmental region of the wing, but through an interaction of this male tissue with female tissue somewhere else. To determine if there is a strong polarized effect associated with the early death focus, the same curve-fitting analysis used above for individual landmarks, was carried out for selected pairs of landmarks. For example, P, D and M were estimated for those mosaics with the humerus male and the third tergite female on the same side. The most striking and consistent feature resulting from this analysis, in all four experiments, was an anterior-posterior polarization such that

Vol. 16 1981

there were about three times as many early deaths in mosaics female anterior to, and male posterior to, a line on the surface of the blastoderm running dorsoventrally between the humerus and third tergite and between the first and third legs. This line was especially well-defined and narrow for Hk^1, which responds best to the curve-fitting analysis. This polarization would explain why early death has its male tissue focus in the region of the wing, which is posterior to this line, and probably means that early death results from deleterious juxtapositions of male and female tissue across the dividing line, rather than only the presence of male tissue in the wing region.

The "mosaic effect" contour maps do not agree with one another so it is difficult at this point to draw any general conclusions from them. It is not possible to compute sturt distances from the computer estimates of *M* (the percent reduction in lifespan of each mosaic type, after compensation for the mutant and early death effects) so the numbers on the mosaic effect maps were obtained by subtracting *M* for mosaics with a given landmark female on both sides, from that when male. Therefore, a positive value indicates a deleterious effect of male tissue, and a negative value, of female tissue. There is no obvious relation to the early death focus but one would expect that this effect as well would be the result of interactions between male and female tissue. With further refinement of the analysis these maps may become more understandable. It is tempting to seek here the reason why male animals are as a rule shorter lived than females, but as reviewed by Lamb (1978) there is no consistent relation between male and female lifespan in *Drosophila* stocks, and indeed, in the present experiments, the male controls lived slightly longer than the females.

Finch (1973) and Comfort (1974) have pointed out that one of the most fundamental, and as yet unanswered, questions in experimental gerontology concerns the location of the pacemakers of aging. The pacemaker, or aging center, connotes mechanisms which regulate the time sequence of physiological and biochemical changes which occur normally during the aging process. It is unknown whether pacemakers reside within each cell or tissue or whether there could be a specific population of cells which directs the course of aging in the other cells and tissues of the organism.

Drosophila would seem to offer the ideal experimental approach for an answer to this question, providing, as it does, a system in which mutants affecting the lifespan of a higher organism may be uncovered, and analyzed using the mosaic technique to determine the developmental focus involved. One of the first mosaic analyses by Hotta and Benzer (1972) was, in fact, of the mutant *drop-dead (drd)* which lives less than a week after eclosion, undergoing an extraordinarily rapid degeneration of the brain tissue. The focus is submissive and maps to the brain. This could, perhaps, be considered an aging mutant with an extremely rapid rate of senescence in the brain tissue. The results presented here for Hk^1 and Sh^5 indicate that both mutant aging effects are associated with the thoracic ganglion, so they may also be neurological problems. This is consistent with the other behavioral abnormalities of these mutants, which are also neurological in origin. It is possible, then, that it is this focus in the thoracic ganglion which is responsible for the increased physical activity and metabolic rate associated with these mutants, and which may be responsible for the decrease in lifespan. If the effect is in fact through metabolic rate, the focus may indeed be a major "aging center" which normally regulates the rate of aging and senescence. At the present time, however, it is still possible that the effect on lifespan could be through stress, or through a lesion having no special relation to the aging process. It is in this sense that the choice of mutants in a study of this kind is critical. It

would be a matter of great interest to make use of other lifespan mutants in an analysis exploiting the mosaic technique in order to determine whether other foci will come to lie in the thoracic ganglion and whether it may eventually be possible to uncover a precisely defined focus. The curve-fitting analyses of *Hk*[1] and *Sh*[5] show that it is feasible to map developmental foci associated with aging, even when the range of mutant lifespans overlaps the normal. This situation is likely to obtain for many other mutants of importance which must be analyzed to unravel the genetic control of the aging process.

SUMMARY

The shaker mutants *Hk*[1] and *Sh*[5] affect the nervous system and reduce lifespan, perhaps through activity-related metabolic rate. Three hundred *y w Hk*[1], *y w Sh*[5], and *y w* mosaics were generated by an unstable ring-X, scored for external male and female tissues, and kept singly to determine the lifespan of each. Because the mosaics did not resolve themselves into distinct long- and short-lived categories, a computer curve-fitting method was developed to transform and combine the appropriate non-mosaic male (short-lived) and female control curves of percent dying per day, into an approximation of the curves observed for mosaics with a given cuticular landmark male on both, one, or neither side. The resulting estimates of long- and short-lived mosaics of each type were used to calculate the distance between each landmark and the developmental focus for *Hk*[1] and *Sh*[5] life-shortening. In both cases the mutant focus was in the ventral anterior part of the thorax, perhaps in the anterior thoracic ganglion; the focus was submissive for *Hk*[1] and domineering for *Sh*[5]. At the same time, there was a second focus, independent of the mutant effect, associated with early mosaic death. In both shaker and control mosaics this early death focus mapped laterally in the region of the wing, and is probably the result of incompatible juxtapositions of male tissue in that region, and female tissue anteriorly. The curve-fitting method may be useful for analyzing other quantitative and multi-focus characters.

Vol. 16 1981

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AGE-RELATED ALTERATION OF THYROIDAL AND MESENTERIC MAST CELLS OF MALE ALBINO RAT

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INTRODUCTION

NUMEROUS WORKERS have described that mast cells are present in the various parts of the body. The recent work of Dasgupta and Maiti (1980) shows that the distribution of mast cells are different in different regions of the body. It is also well established that the mast cells play a role in controlling local circulation by providing local hormone (Jaques, 1978). The quantities of histamine and heparin in mast cells are relatively enormous (Jaques, 1975). In addition to histamine and heparin, some mast cells also contain serotonin (Benditt and Lagunoff, 1963). Cyclic changes in the number of mast cells in tissues or organs such as uterus or in the skin of man and various mammalian species, as well as age dependent variations, have been described by several authors (Bates, 1935; Padawer and Gordon, 1956; Padawer, Massland, and Pankove, 1961; Simpson and Hayashing, 1960, and Smith, 1958). Okudaira *et al.* (1980) observed pronounced difference of the mast cell count of the peritoneal fluid and histamine content in varied body weight of male rats. It is well-known that the activity of thyroid gland is greater in a younger one than that of an older one. Dasgupta and Maiti (1978) observed the changes in the mast cell count in experimentally induced hyper and hypoactive thyroid gland.

According to David *et al.* (1976) plasma TSH and T_4 concentration is altered during different phases of the oestrous cycle. The role of TSH and TRF level of the body on the thyroidal mast cell population was observed by Gall (1974). The present study has been designed to explore the changes in the thyroidal mast cell population in relation to age, and take mesentery as a non-target organ where we can avoid the effect of interaction of different hormones on mast cell population.

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METHODS AND MATERIALS

Laboratory bred Holtzman strain rats of same littermates of different age groups were distributed at random into eight groups of three animals each, according to their body weights. Emphasis was given regarding the selection of animals in relation to body weight with age.

Group I = 3 male rats of 50–60 g of body weight (age 5 weeks)
Group II = 3 male rats of 100–110 g of body weight (age 9 weeks)
Group III = 3 male rats of 142–150 g of body weight (age 14 weeks)
Group IV = 3 male rats of 198–210 g of body weight (age 18 weeks)
Group V = 3 male rats of 247–257 g of body weight (age 21 weeks)
Group VI = 3 male rats of 300–320 g of body weight (age 26 weeks)
Group VII = 3 male rats of 347–365 g of body weight (age 30 weeks)
Group VIII = 3 male rats of 434–450 g of body weight (age 42 weeks)

The animals were fed a standardized pellet diet (Hindusthan Lever & Co.) with tap water *ad libitum*. All animals were killed by decapitation. Two lobes of thyroid from the animals were collected, weighed and fixed in formol-alcohol solution. Retroperitoneal connective tissue of duodenum and the mesentery of ileum of all the animals were taken and preserved as follows. A middle line incision of the abdominal wall was made. The intestine and mesentery were removed and the mesentery of the small intestine were carefully spread upon a glass slide. The material was fixed in 10% neutral formalin-alcohol (1:4) solution for 24 h. After fixation the attached intestine was cut away with small scissors carefully avoiding stretching of the membrane which was placed on the microscopic slide. The specimens were stained with 0.1% toluidin blue solution and the number of mast cells in membranous, fatty and perivascular areas were counted under microscope. Thyroid was cut in cryo-cut microtome (−20°C) at 15μ thickness and stained with 0.1% toluidin blue solution. The mesenteric mast cells were counted under the light microscope at a magnification of 150×. The sections of the thyroids were studied under light microscope at a magnification of 600×. The numbers of mast cells per field were counted considering at least 20 sections from each thyroid gland. The countings were based on their morphological appearance as intact and degranulated mast cells:

1. Intact (I): where the cell membrane was intact.
2. Degranulate (D): where the cell membrane became reputured.

RESULTS

Table 1 shows the weights of the thyroid glands and the average number and morphological variations of the mast cells in the mesentery and thyroid glands in the animals of different age groups. From the table it was found that there was a tendency of increased thyroid weight with age, but if the weight was expressed in terms of 100 g of body weight, the thyroid weight decreased with age. The mast cells count was also found to be varied significantly among the different groups of animals. Intrathyroidal mast cell numbers were found to be decreased with increasing age. The numbers of intact cells were found to be greater in the younger group of rats than those of the older one. But, the morphological variation, the total degranulations were more prevalent in the older group. On the contrary, in case of mesentery the population of mast cells was greater in the distal portion of the small intestine. i.e., ileum. The mast cells are generally observed more in the perivascular fatty tissue in the duodenum than that of the membraneous portion, whereas in case of ileum this picture is not well manifested.

DISCUSSION

The results of different workers reveal that the distribution of mast cells is influenced by age and sex of animals. The present report also suggests that the number of mast cells changes with age in different tissues. Okudaira *et al.* (1980) observed the increased number of mast cell count in peritoneal fluid of rat and also found correlation between the number of mast cell and histamine content. Helistoom and Holmgren (1950) compared

Vol. 16 1981

TABLE 1. AVERAGE NUMBER AND MORPHOLOGICAL VARIATIONS OF MAST CELLS PER FIELD UNDER LIGHT MICROSCOPE

Body wt. in g	Age (in Wks.)	Thyroid wt. in mg	Thyroid wt. per 100 g B.W. (in mg)	No. of mast cells/per field			% morphological variation of mast cells in thyroid	
				Thyroid	Mesentery			
					Duodenum	Ileum		
				× 600	× 150	× 150	Intact	Degranulation
50–60 (3)	5	5.60 ± 0.6	11.20 ± 0.6	5.0 ± 0.7	22.8 ± 3.5	33.7 ± 4.2	80.0	20.0
100–110 (3)	9	12.6 ± 1.7	12.6 ± 1.7	5.6 ± 1.05	31 ± 6.7	60 ± 12.0	73.4	26.6
142–150 (3)	14	13.4 ± 1.8	9.0 ± 1.2	6.1 ± 0.5	39 ± 5.6	72.6 ± 11.4	74.3	25.7
198–210 (3)	18	16.1 ± 2.5	8.0 ± 1.25	4.8 ± 0.19	48 ± 10	81 ± 9.6	70.1	29.9
247–257 (3)	21	17.5 ± 2.4	6.8 ± 0.96	4.3 ± 0.26	52.6 ± 5.0	85 ± 8.2	65.4	34.6
300–320 (3)	26	15.8 ± 1.9	5.3 ± 0.63	3.9 ± 0.37	56.0 ± 7.25	96 ± 7.8	58.7	41.3
347–365 (3)	30	14.7 ± 1.6	4.2 ± 0.46	3.6 ± 0.35	64.6 ± 7.6	98.1 ± 10.6	53.2	46.8
434–450 (3)	42	16.1 ± 1.1	3.6 ± 0.2	3.4 ± 0.13	58.3 ± 6.5	65.8 ± 8.2	48.1	51.9

Values are mean ± *SD*. The number of animals is in parenthesis.

mast cell population increase with the age of human skin and heart among the different ages, and found that this number significantly dropped with advancing age. Lindholms (1954) observed similar changes of the mast cells with age in the wall of alimentary canal. In contrast to the above observation, Marx *et al.* (1960) reported an increase in the mast cells population in liver, kidney, lung and thymus of rat with age. From our results it is clear that the mast cells of the mesentery increased with age and their distribution also varied. The functional significance of the mast cells in the different regions of the intestine cannot be elucitated by the present experimental condition. But it is suggested that the greater population of mast cells in the perivascular fatty tissue in duodenum and thin membranous mesentery of ileum may be related to the increased vascular permeability caused by histamine liberated from the mast cells. To some extent, it is known that tissue histamine (Schayer, 1963) markedly increases the vascular permeability and thus results in greater lymph flow in response to fat diet for fat transport (Wollin and Jaques, 1973; Wollin, 1973). This conclusion is further reinforced by experiments on mast cells in rats (Jaques, 1978).

In contrast to the increase number of mast cells in the mesentery, the intrathyroidal mast cells decrease with increasing age. It is well-known that a younger individual has a higher BMR than that of the older one. Among the many endocrine and non-endocrine factors affecting BMR, age, sex, environmental factors, thyroidal hormone and CNS activity are important. It has been reported (Beltz and Reinete, 1968) in rats that thyroid activity is low at birth. Up to 10 days after that it begins to increase. Other observers (Gregerman, 1963; Johnson, Ward, and Kibler, 1966) also reported the unchanged secretion of thyroid hormone up to 2–3 months which begins to decline from 2–3 months to middle life for about one year. Von Zür Mühlen (1977) observed that TRF induced TSH release is decreased with increasing age. Nunez and Gershon (1973) have shown that mast cells are present in thyroid glands of a number of species and are related to the regulation of thyroidal activity. Malandar (1971) has shown in mice that administration TSH increases the number of mast cells in the thyroid gland and also induces the release of biogenic amines and metachromatic materials from the thyroidal mast cells. In rats, 5-HT and histamine are present in the thyroidal mast cells within the bound granules (Clayton and Szego, 1967). Gall *et al.* (1974) observed that the effect of TRH on the mast cells was mediated via TSH. Dasgupta and Maiti (1978) have also shown that the mast cell population depends on TSH under different altered function of the thyroid gland, confirming the observation of others (Clayton *et al.*, 1967; Malander *et al.* 1971; Gall *et al.*, 1974).

From our results it is suggested that a certain relationship exists between the number thyroidal and mesenteric mast cells with age. The decrease in the number of the thyroidal mast cells with age supports the hypothesis of Wilansky *et al.* (1957) who suggests the decreased activity of thyroid with age. From the study of Rapp and Lilha (1974) it is revealed that there might be an inverse relationship between the plasma TSH level with aging. So this decrease in the plasma TSH in turn might decrease the mast cell number in the thyroid gland.

The significance of the increased number of mast cells in mesentery with age cannot be correlated at this stage but it may be suggested that their presence in profuse numbers in intestines with age may be related to (a) increased lymph flow causing fat absorption partly (Wollin & Jaques, 1973, 1976), (b) ion-exchanger function (Padawer, 1974) and (c) local circulatory regulator, i.e., microcirculation (Schayer, 1962).

SUMMARY

Mast cell count of the thyroid and the mesentery in adult male rats of varying body weight and of different ages was studied by toluidine blue staining method. Mast cell numbers in the thyroid gland were found to be decreased with age but these changes were reversed in case of mesentery. Results show the possible co-relation between the thyroidal mast cells and thyroidal activity with age.

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Vol.
16
1981

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EFFECT OF AGE AND COLD SHOCK ON THE SDH ACTIVITY OF BRAIN MITOCHONDRIA AND CARBOHYDRATE CONTENTS OF BRAIN OF MALE GARDEN LIZARD

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INTRODUCTION

TWO SYSTEMS, nervous and endocrine, play pivotal roles in regulation and integration of the relationship of the organisms to the environment. Selye (1950) recognized the central role of hypothalamo-pituitary-adrenocortical system in the response of organism to environmental influences and derived the term "general adaptation syndrome." It is generally believed that physiological adaptations to environmental changes alter during ageing. This led to the concept of stress theory of ageing proposed by Selye and Prioreschi (1960).

Reptiles are more resistant to extremes of cold than of heat (Bellairs and Attridge, 1975). Prolonged exposure to temperature below freezing point is probably lethal for them but above this level they can exist in a state of suspended activity for long periods. This level has been termed as "critical minimum temperature" or CMT by Spellerberg (1976). In temperate species of reptiles the CMT may go down to −9°C. Discussing the problems of adaptation of reptiles to cold Spellerberg (1976) reported that in some temperate species of reptiles there may be the presence of a super-cooling system (a state where tissue freezing has not occurred yet the animal core body temperature is below the freezing point of the blood) as adaptation to cold. The same author also suggested that studies on adaptive alteration at the metabolic level of reptile subjected to the induction of low body temperature be undertaken for a better assessment of the problem of cold adaptation of reptiles.

In our laboratory we have examined some aspects of metabolic changes in the tissues of the male garden lizard, *Calotes versicolor*, in adaptation to cold. Earlier two series of experiments (Das and Patnaik, 1979, 1980) were done and it was observed that the garden lizards when exposed to a temperature range of 0–4°C for one hour remain inactive possibly due to cold narcosis as suggested by Bogert (1959). During this treatment their body temperature (rectal) fell almost to the level of surrounding temperature. When

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brought to room temperature they recovered to normal stage. Metabolic studies revealed that there was a decline in O_2 consumption of mid-brain homogenates of garden lizards following cold exposure and this was age-dependent. It was also shown that the recovery in O_2 consumption when brought to room temperature after cold exposure was achieved early in young than in old lizards. The depression in SDH activity of mid-brain homogenates following cold-exposure was, however, not found to be age-dependent.

To get further insight into the problem of age-dependency of adaptation of reptiles to cold, attempts have been made in the present series to evaluate the effect of short-term cold shock on some metabolic changes in the brain of the garden lizard, *Calotes versicolor*.

MATERIALS AND METHODS

The lizards were collected from nature during February-April for studies on enzyme activity and during September-December for the estimation of biochemical constituents. They were maintained at room temperature for a week before use and a diet of goat liver and water was provided *ad libitum*. Age-grouping was based on the body weight and S-V length of lizards. Actual ages of lizards were determined by bone histology (Patnaik and Behera, 1981). While the age of "young" lizards was under 1 year the "middle-aged" and "old" were 1 and 2–4 years old respectively.

Lizards were subjected to cold-shock treatment of shorter duration as done by Saha and Pramanik (1977). In this process the hind limbs of the lizards and the tail up to their origin were immersed in ice cold water (0–5°C) for 5 minutes and then used either for the study of SDH activity in mitochondrial pellet or biochemical constituents of brain in young, middle-aged and old groups of lizards. Simultaneously, matched controls maintained in laboratory were also used for comparison.

Mitochondria from the brain were isolated following the method as described by Das, Kara and Patnaik (1979). SDH activity was determined by Kun and Abood's (1949) method and the protein content by the method of Lowry *et al.* (1951). For the estimation of biochemical constituents, the brain was divided longitudinally into two halves with a sharp razor blade. One half was used for the estimation of glucose (Mendel, Kemp, and Myers, 1954) and the other for glycogen (Kemp, Kits, and Van Heijningen, 1954). Glucose levels of blood drawn from the lightly etherized animals were estimated following the method of Mendel, Kemp, and Myers (1954) after treating the sample with deproteinizing solution. Whenever necessary blood and tissue samples from two lizards of approximately same size were pooled to procure adequate material for biochemical analysis.

RESULTS AND DISCUSSION

Our data on normal age changes in lizard brain revealed that the SDH activity reached a peak level in the middle-aged group and showed a decline there-after (Table 1). This decrease in respiratory metabolism might be due to a loss of functional integrity of mitochondria as has been suggested for mammals by Wilson and Franks (1975). Loss of SDH activity in brain has also been reported for mammals (Meier-Ruge *et al.*, 1976).

Brain tissue is almost completely dependent on carbohydrates for its energy source (Bachelard, 1970). Glucose has been confirmed to be the only satisfactory metabolic support for the brain *in vivo*. In garden lizards both glucose and glycogen contents of brain show only a marginal rise with maturation and ageing concomitant with the trend exhibited by blood glucose level (Table 2). That under resting conditions there is no change in blood sugar level with advancing age of human beings (Shock, 1968) and only a little change in glucose content of mouse brain (Maker *et al.*, 1973) have been reported earlier.

Short-term cold shock led to a rise in mitochondrial SDH in the brain of lizards of all the age-groups (Table 1). While in young and middle-aged the rise was only marginal, in old it was highly significant.

Selye (1950) recognized three phases in the response of organisms to stress. These are "alarm reaction" occurring during initial phase, a "stage of resistance" where there is an enhanced adaptive capacity and a "stage of exhaustion" where there is a loss of capacity to

TABLE 1. EFFECT OF COLD SHOCK ON THE MITOCHONDRIAL SDH ACTIVITY OF WHOLE BRAIN OF MALE GARDEN LIZARDS OF THREE DIFFERENT AGE GROUPS

Age group	Experimental condition	Range of body wt. (g)	Range of S-V length (cm)	SDH activity-μg formozan formed/ mg. protein/hr. (Av ± S.E.M.)	*p*	% increase
	Control	5–15	6.8–9.0	8.68 ± 1.91 (5)		
Young					NS	9.6
	Cold shocked	6–15	7.0–9.0	9.52 ± 0.95 (6)		
p				NS		
	Control	23–40	10.2–11.4	11.35 ± 1.45 (8)		
Middle-aged					NS	1.7
	Cold shocked	22–40	9.9–11.8	11.55 ± 2.20 (9)		
p				< 0.05		
	Control	45–65	11.5–13.7	6.82 ± 0.33 (6)		
Old					< 0.02	40
	Cold shocked	45–61	11.3–12.1	9.55 ± 0.96 (5)		
p				NS		

Note: *p* values represented horizontally refer to comparison among subsequent age-groups whereas those represented vertically refer to comparison between control and cold shocked of same age-group. Numbers in parentheses indicate sample size; percent change calculation was based on average values.

Vol. 16 1981

adapt. The rise in SDH activity of the brain mitochondria of garden lizard during cold exposure seems to be due to "alarm reaction." Saha and Pramanik (1977) also observed a rise in the activity of another mitochondrial membrane bound enzyme (Monoamine oxidase) of the brain of lizard following short-term cold shock. A rise in brain cholinesterase activity of rat brain following cold stress during "alarm reaction" has also been reported by Naik *et al.* (1977). Even though adequate evidence from poikilothermic vertebrates is lacking (Hazel and Prosser, 1974), it appears that in mammals the hypothalamo-pituitary-adrenal and hypothalamo-pituitary-thyroid axes are stimulated during cold exposure. It is also known that the neurosecretion in garden lizard is temperature-dependent (Banerjee and Das, 1969; Vijayakumar *et al.*, 1980). However, it remains to be decided as to whether the changes observed in SDH activity during cold shock is mediated through some such systems or is a direct action on CNS.

Blood glucose level and glucose and glycogen contents of brain declined following cold shock treatment (Table 2). That the biochemical composition of blood (Stanton and Mueller, 1973) and brain (McIlwain and Bachelard, 1971) of mammals change in response to stress has been reported earlier.

The rise in SDH activity of the brain of old lizards following cold shock was significantly higher than that of young and middle-aged. Cold shock induced a greater percentage of decrease in glucose and glycogen contents of brain of old than in young and middle-aged lizards even though the blood glucose level showed a reverse age-related trend. Increased utilization of glucose and glycogen during cold shock seems to be more promi-

TABLE 2. EFFECT OF COLD SHOCK ON BLOOD GLUCOSE LEVEL AND CARBOHYDRATE CONTENTS OF BRAIN OF MALE GARDEN LIZARDS OF THREE DIFFERENT AGE GROUPS

Age group	Experimental condition	Range of body wt. (g)	Range of S-V length (cm)	mg. glucose/100 ml blood	p	% decrease	mg. glucose/g wet wt.	p	% decrease	mg. glyco gen/g wet wt.	p	% decrease
	Control	5–15	6.2–9.4	188.196 ± 20.149 (6)			1.037 ± 0.118 (8)			0.464 ± 0.041 (8)		
Young					< 0.1	32		NS	14		< 0.05	31
	Cold shocked	5–15	6.6–9.4	126.331 ± 16.593 (5)			0.889 ± 0.139 (7)			0.320 ± 0.044 (7)		
p				NS			< 0.02			NS		
	Control	21–40	9.4–12.1	226.471 ± 19.248 (7)			1.750 ± 0.203 (9)			0.574 ± 0.056 (9)		
Middle aged					< 0.05	27		< 0.1	31		< 0.01	40
	Cold shocked	20–38	9.3–11.9	164.248 ± 15.789 (6)			1.207 ± 0.123 (7)			0.339 ± 0.029 (7)		
p				NS			NS			< 0.1		
	Control	45–60	11.7–13.2	219.548 ± 17.370 (7)			2.116 ± 0.282 (8)			0.807 ± 0.103 (9)		
Old					NS	7		< 0.1	29		< 0.01	47
	Cold shocked	46–65	11.2–13.3	204.09 ± 6.809 (6)			1.489 ± 0.108 (8)			0.422 ± 0.027 (8)		

Note: p values represented horizontally refer to comparison among subsequent age-groups whereas those represented vertically refer to comparison between control and cold shocked of same age-group.

Values are average ± SEM. Numbers in parentheses indicate sample size; percent change calculation was based on average values.

nent in old than in young lizards. Since glycogen synthetase or phosphorylase activities have not been tested, the decline in glycogen content can not be entirely attributed to accrue from utilization during cold shock treatment. However, assuming that the changes are mainly due to degradation of glycogen rather than to impaired synthesis, the results suggest a lesser adaptive ability of the old lizards to cold shock as there is a greater expenditure of energy. Reptiles do not utilize metabolic heat for temperature regulation. They produce relatively less heat and whatever they produce is easily lost. It is likely that in the brain of old lizard the activation of SDH during short-term cold shock treatment leads to an increased metabolic activity. Some amount of heat which is produced during this metabolic activity is probably lost to the environment. The present findings therefore partially support the implications involved in the stress theory of ageing that the physiological adaptation alters with advancing age. Further work on similar lines is in progress to derive additional evidences.

SUMMARY

The SDH activity of brain mitochondria reached a peak level when the lizards were 1 year old and then declined. Blood glucose level and the carbohydrate contents (glucose and glycogen) exhibited a trend of rise with advancing age.

Short term cold shock induced a significant rise in mitochondrial SDH activity in old lizards but only marginally in young and middle-aged. While the degree of decline in glucose and glycogen contents of brain of old following cold shock was significantly higher than that of young, the fall in blood glucose level followed a reverse trend.

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Vol. 16 1981

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AMINOACYLATION OF tRNA BY CELL-FREE PREPARATIONS FROM AGING *DROSOPHILA MELANOGASTER*

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INTRODUCTION

AN AGE-RELATED decrease in protein synthesis has been reported to occur in adult *Drosophila melanogaster* (Baumann and Chen, 1968; Chen, 1972) and *Drosophila subobscura* (Maynard-Smith *et al.*, 1970). In agreement with these observations on intact organisms, we found that protein synthesis by a microsomal preparation from *Drosophila melanogaster* declined approximately 70% during the first half of adult life and continued a slow decrease thereafter (Webster and Webster, 1979). In view of the potential importance of a decrease in protein synthesis for the observed age-related deterioration of cellular structure and function in *Drosophila* (Takahashi *et al.*, 1970; Miquel, 1971; Gartner, 1973; Baker and Schmidt, 1976; Vann and Webster, 1977; Webster and Webster, 1978; Miquel and Johnson, 1979), it is of considerable interest to know the molecular basis for the decrease.

Protein synthesis can be divided operationally into four major stages: aminoacylation of tRNA; peptide chain initiation; peptide chain elongation; and termination. An important question concerns which stage or stages deteriorate to produce the observed decline in protein synthesis. Since the aminoacylation of tRNA is prerequisite to the other stages in protein synthesis, information on the possible age-related deterioration of this process is a necessary first step in understanding the molecular basis of the decrease in protein synthesis.

Decreased capacity for aminoacylation of tRNA has been observed in cell-free preparations of aging mouse liver (Frazer and Yang, 1972) and rat liver (Mays *et al.*, 1979; Lawrence *et al.*, 1979). The effect of age on the capacity for aminoacylation of tRNA by nine different amino acids was studied in extracts of aging *Drosophila melanogaster* by Hosbach and Kubli (1979). During the period when we observed the greatest decrease in protein synthesis (Webster and Webster, 1979), they found that there was little or no decrease in the amount of aminoacylation of tRNA by glycine, isoleucine, threonine,

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valine, proline, alanine, or serine, but approximately a 60% decrease in the amount of arginine attached to tRNA and a 53% decrease in the amount of leucine attached to tRNA. Although decreases in capacity for aminoacylation are not directly comparable with a decreased rate of protein synthesis, the findings of Hosbach and Kubli (1979) make it of considerable interest to know whether there is a decrease in either the rate or capacity of aminoacylation that is sufficient to act as a possible limiting factor in protein synthesis. We have, therefore, examined the effect of age on both the rate and extent of aminoacylation of tRNA in the cytosol of *Drosophila melanogaster*.

MATERIALS AND METHODS

Drosophila melanogaster, strain Oregon R, was grown on a cornmeal medium (Samis *et al.*, 1971) at 20° and 60% relative humidity. Groups of approximately 50 flies were housed in polycarbonate jars with plastic sponge stoppers. Cultures were transferred each week to fresh medium. Unlike the case with mammals, the life-span of *Drosophila* is rather sharply divided into developmental (larval and pupal) and adult stages. Out of a total life-span of approximately 57 days, the developmental stages occupied the initial 14 days and the adult stage the remaining 43.2 days. Most of the cells of adults are postmitotic and age uniformly. The life-spans expressed in this paper are for the adult stage.

The cell-free preparation used for assay of aminoacylation of tRNA was prepared by a modification of the method used by Rose and Hillman (1969). One gram of flies was immobilized by chilling to approximately 3°, and was homogenized in a power-driven glass homogenizer with nine ml of a medium consisting of 0.035 M tris(hydroxymethyl)aminomethane, 0.025 M KCl, 0.01 M $MgCl_2$, 0.007 M mercaptoethanol, and 0.005 M phenylthiourea. The medium was adjusted to pH 7.6 before use. The preparation was centrifuged at 20,000 × g for 20 min, and the sediment was discarded. The supernatant fluid was centrifuged at 27,000 × g for 20 min, and the sediment was discarded. The supernatant fluid was centrifuged at 105,000 × g for 120 min. The sediment was discarded, and the supernatant solution was dialyzed for 18 hours against 0.01 M tris(hydroxymethyl) aminomethane (pH 7.6)-0.005 M mercaptoethanol.

The assay system for aminoacylation of tRNA was that of Rose and Hillman (1969) and, unless otherwise specified, consisted of: 0.003 M imidazole (pH 7.0), 0.001 M $MgCl_2$, 0.0024 M sodium ATP, 0.0024 M dithiothreitol, 0.1 μmole of amino acid containing approximately 1 μCi of radioactivity, and 0.3 ml of high-speed supernatant fluid (containing approximately one mg of protein and 0.43 A_{260} unit of tRNA) in a total volume of 0.5 ml. The reaction was started by addition of the supernatant protein solution, and proceeded at 30° for the times indicated. The reaction was stopped by pouring the reaction mixture into five ml of 10% trichloroacetic acid on a 0.45 μm membrane filter. The mixture was filtered with vacuum, and the precipitate on the filter was washed with a total of 40 ml of 10% trichloroacetic acid. The filter was dried at 80° for 20 min and immersed in 20 ml of Aquasol-2® (New England Nuclear Corp., Boston, Mass.). Radioactivity was determined in a Beckman liquid scintillation counter. The values given are the means of four measurements. The radioactive amino acids used were: ^{3}H-L-alanine (16.1 Ci/mmole), ^{3}H-L-arginine (18.1 Ci./mmole), ^{14}C-L-asparagine (0.2 Ci/mmole), ^{3}H-L-aspartic acid (15.8 Ci/mmole), ^{3}H-L-glutamic acid (17.5 Ci/mmole), ^{3}H-glycine (15 Ci/mmole), ^{3}H-L-histidine (2 Ci/mmole), ^{3}H-L-isoleucine (104.5 Ci/mmole), ^{3}H-L-leucine (5 Ci/mmole), ^{3}H-L-lysine (2.7 Ci/mmole), ^{3}H-L-methionine (0.2 Ci/mmole), ^{3}H-L-phenylalanine (9.2 Ci/mmole), ^{3}H-L-proline (2.5 Ci/mmole), ^{3}H-L-serine (2.8 Ci/mmole), ^{3}H-L-threonine (2.0 Ci/mmole), ^{3}H-L-tryptophan (7.3 Ci/mmole), ^{3}H-L-tyrosine (51 Ci/mmole), and ^{3}H-L-valine (2.1 Ci/mmole), all obtained from New England Nuclear Corp.; ^{14}C-DL-cysteine (15 mCi/mmole) obtained from Schwarz/Mann, Orangeburg, N.Y.; and ^{14}C-L-glutamine (45 mCi/mmole) obtained from ICN Pharmaceuticals, Irvine, Calif. The ^{3}H-amino acid mixture consisted of: L-alanine, L-arginine, L-aspartate, L-glutamate, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, and L-valine, and had a specific activity of 0.26 Ci/mmole. The procedure described by Rose and Hillman (1969) was used for tRNA preparation, and protein was measured by the biuret method of Gornall *et al.* (1949). The *Drosophila* enzymes were able to aminoacylate yeast tRNA (Type III, Sigma Chemical Co., St. Louis, Mo.) at rates equal to the tRNA of young flies.

RESULTS

Aminoacylation by cytosol preparations

With the exception of small amounts bound to ribosomes, most of the aminoacylation enzymes and tRNA are in the cytosol. In assessing the possible contribution of decreased

aminoacylation to the observed age-related decrease in protein synthesis, it is important to know the effect of age on aminoacylation in the cytosol. Any age-related changes in either the aminoacylation enzymes or in the tRNA should be reflected in the rates or extents of aminoacylation in the cytosol.

Fig. 1 presents the time course of aminoacylation by a mixture of 15 ^{3}H-amino acids of tRNA in the cytosols prepared from adult flies of increasing age. The initial rate of aminoacylation by 21-day-old adults, an age where the rate of cell-free protein synthesis

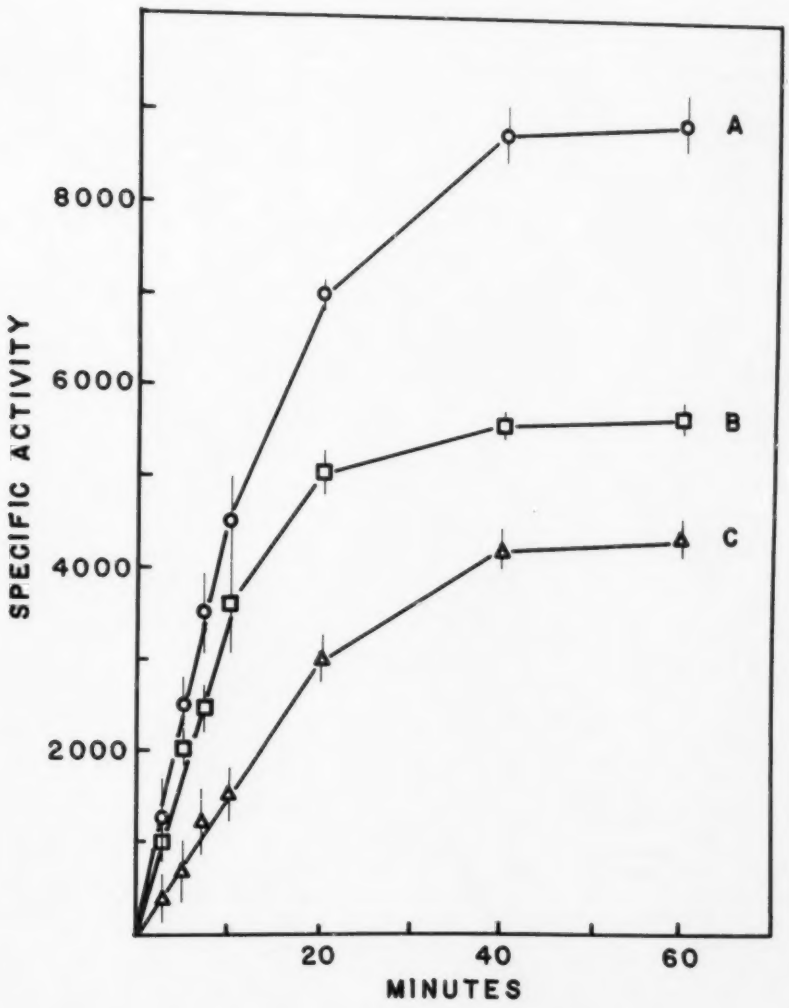

FIG. 1. Time course of aminoacylation of tRNA by a mixture of 15 ^{3}H-amino acids in the cytosol of 1-day-old adults (a), 21-day-old adults (b), and 42-day-old adults (c). Reaction system and amino acids comprising the mixture are described in the Materials and Methods section. Specific activity equals pC ^{3}H-amino acid mixture attached to tRNA by 1 mg of cytosol protein.

Vol. 16 1981

had declined 70% (Webster and Webster, 1979), did not decrease by more than 20%. However, in the cytosol of 42-day-old adults, which is approximately their mean life-span, the rate of aminoacylation decreased approximately 66%.

In contrast to the lack of decrease in the initial rate of aminoacylation in 21-day-old flies, the extent of aminoacylation diminished approximately 36%. The extent of amino-acylation decreased 50% in 42-day-old flies as compared with 1-day-old adults.

Although the overall rate of aminoacylation by a mixture of 15 amino acids did not parallel the decreased protein synthesis in 21-day-old adults, aminoacylation could contribute markedly to the drop in protein synthesis if the rate or extent of aminoacylation of only one amino acid should exhibit an age-related decrease to zero. Table 1 presents the initial rates of aminoacylation of tRNA by each of the 20 amino acids in the cytosols of one-day-old and 21-day-old flies. Aminoacylation rates increased or exhibited no change with 12 of the amino acids and decreased between 9% and 38% with the other eight amino acids. Table 2 presents the total extent of aminoacylation of the tRNA in the cytosols of 1-day-old and 21-day-old flies. Although the extent of aminoacylation increased in the case of four amino acids, it decreased between 2% and 54% with the remaining 16 amino acids. Although the sums of the rates or extents of aminoacylation by the individual amino acids cannot be compared directly with aminoacylation by the amino acid mixture because of concentration differences, the general trends with the individual amino acids are similar to those exhibited by the amino acid mixture. However, none of the amino acids exhibited a decrease in rate or extent of aminoacylation that was comparable with the age-related decrease in protein synthesis.

TABLE 1. CHANGES IN THE RATES OF AMINOACYLATION OF tRNA BY INDIVIDUAL AMINO ACIDS DURING THE PERIOD OF MAXIMAL DECREASE IN THE RATE OF MICROSOMAL PROTEIN SYNTHESIS

Amino acid	Specific activity*		21-day-old/ 1-day-old ratio
	1-day-old adults	21-day-old adults	
Alanine	0.45 ± 0.05	0.45 ± 0.06	1.00
Arginine	0.50 ± 0.04	0.67 ± 0.06	1.34
Asparagine	0.67 ± 0.18	0.50 ± 0.11	0.75
Aspartate	0.90 ± 0.12	1.71 ± 0.20	1.90
Cysteine	5.88 ± 0.78	5.27 ± 0.77	0.90
Glutamate	7.58 ± 0.93	4.66 ± 0.65	0.62
Glutamine	0.36 ± 0.04	0.56 ± 0.04	1.56
Glycine	1.50 ± 0.11	1.98 ± 0.17	1.32
Histidine	0.22 ± 0.01	0.20 ± 0.02	0.91
Isoleucine	1.46 ± 0.17	1.29 ± 0.11	0.88
Leucine	0.94 ± 0.10	1.24 ± 0.13	1.32
Lysine	2.43 ± 0.31	3.59 ± 0.45	1.48
Methionine	2.76 ± 0.30	1.70 ± 0.23	0.62
Phenylalanine	1.79 ± 0.21	2.96 ± 0.26	1.65
Proline	2.25 ± 0.25	3.28 ± 0.40	1.46
Serine	0.49 ± 0.33	0.76 ± 0.48	1.55
Threonine	0.27 ± 0.22	0.41 ± 0.29	1.52
Tryptophan	1.83 ± 0.21	1.60 ± 0.15	0.87
Tyrosine	2.08 ± 0.25	1.58 ± 0.14	0.76
Valine	0.45 ± 0.50	0.53 ± 0.31	1.18

*p moles amino acid attached to tRNA in 1 min per mg of cytosol protein.

TABLE 2. CHANGES IN THE TOTAL AMOUNTS OF AMINOACYLATION OF tRNA BY INDIVIDUAL AMINO ACIDS IN THE CYTOSOL DURING THE PERIOD OF MAXIMAL DECREASE IN THE RATE OF MICROSOMAL PROTEIN SYNTHESIS

Amino acid	Total aminoacylation*		21-day-old/ 1-day-old ratio
	1-day-old adults	21-day-old adults	
Alanine	33.6 ± 3.5	35.3 ± 3.1	1.05
Arginine	25.9 ± 3.0	23.3 ± 2.8	0.90
Asparagine	13.9 ± 2.9	13.2 ± 1.8	0.95
Aspartate	27.7 ± 3.4	12.9 ± 2.1	0.47
Cysteine	15.8 ± 2.4	14.5 ± 2.7	0.92
Glutamate	87.0 ± 5.0	40.2 ± 4.5	0.46
Glutamine	20.2 ± 3.2	12.7 ± 2.1	0.63
Glycine	30.4 ± 1.5	28.6 ± 1.9	0.94
Histidine	18.0 ± 2.7	15.1 ± 2.0	0.83
Isoleucine	20.0 ± 3.1	20.8 ± 2.2	1.04
Leucine	10.6 ± 2.4	10.4 ± 3.2	0.98
Lysine	35.1 ± 4.0	32.8 ± 6.4	0.93
Methionine	16.1 ± 1.0	14.7 ± 1.5	0.91
Phenylalanine	22.7 ± 3.3	26.7 ± 3.8	1.18
Proline	11.1 ± 1.8	6.5 ± 1.0	0.59
Serine	16.5 ± 2.0	10.6 ± 1.6	0.64
Threonine	40.2 ± 2.8	25.3 ± 1.9	0.63
Tryptophan	24.6 ± 3.0	26.0 ± 1.7	1.06
Tyrosine	20.8 ± 1.1	19.7 ± 1.4	0.95
Valine	34.9 ± 4.3	29.7 ± 3.2	0.85

* *p* moles amino acid attached to tRNA (approximately 0.43 A_{260} units) per mg cytosol protein.

Effect of age on the activity of the aminoacylation enzymes

The age-related decreases in the rates and extents of aminoacylation in the cytosol could be due to deterioration of the aminoacylation enzymes, to deterioration of tRNA, or to a combination of the two. The aminoacylation enzymes in the cytosol exhibited a typical substrate saturation curve for tRNA (Fig. 2a). In the presence of saturating amounts of added tRNA, the rate of aminoacylation was directly proportional to enzyme protein (Fig. 2b). Under conditions of excess added tRNA, cytosol preparations from flies of increasing age showed no decrease in the rate of aminoacylation (Table 3). There was instead an increase in the initial rate. From this, it does not appear that overall aminoacylation enzyme activity deteriorated significantly with age, although a single enzyme might exhibit decreased activity.

Effect of age on tRNA

In view of the lack of deterioration of overall aminoacylation enzyme activity, the decreased extent of aminoacylation in the cytosol of aging organisms (Fig. 1) could be due either to decreased levels of tRNA or to an alteration of tRNA which reduced its ability to accept amino acids. Table 4 presents the levels of tRNA in the cytosols from flies of different ages. It is evident that there was a decrease of about 21% after 21 days and about 25% after 42 days of adult life. Table 4 also presents the ability of tRNA from flies of different ages to accept a mixture of ^{3}H-amino acids. The extent of aminoacylation per absorbance unit decreased significantly in tRNA isolated from flies of increasing age. The

Vol. 16 1981

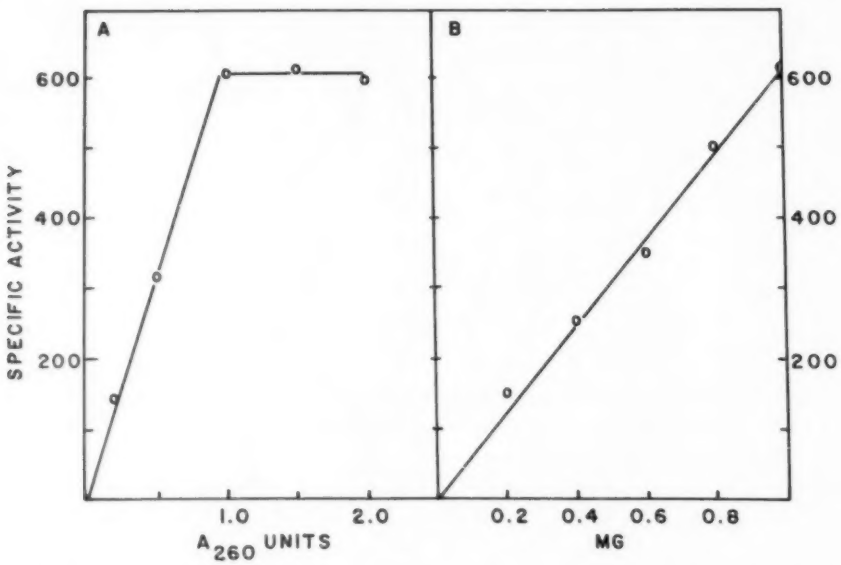

FIG. 2a-b. Effect of concentration of tRNA (a) or enzyme (b) on the rate of aminoacylation. For determination of the effect of substrate concentration, the noted amounts of tRNA (including the 0.43 A_{260} unit already present in the cytosol) were added to the reaction system and the initial rates of aminoacylation were measured. For determination of the effect of enzyme concentration, tRNA concentration was adjusted to 1.5 A_{260} units (including that added with the cytosol) in the presence of the noted amounts of cytosol protein, and the initial rate of aminoacylation measured. Specific activity was pC of ^{3}H-amino acid mixture attached to tRNA per min.

nature of this decreased ability to accept amino acids is not known, but it may be similar to the phenomena previously observed by Mays and colleagues in rats (Lawrence *et al.*, 1979; Mays *et al.*, 1979) and by Hosbach and Kubli (1979) in *Drosophila*.

DISCUSSION

It seems fairly well established that there is a significant, age-related decrease in protein synthesis in adult *Drosophila*. The purpose of this investigation was to determine whether the decrease in protein synthesis might be due, at least in part, to a decrease in amino-acylation of tRNA. However, during the first 21 days of adult life, when the rate of cell-

TABLE 3. EFFECT OF AGE ON THE INITIAL RATES OF AMINOACYLATION OF tRNA BY A MIXTURE OF ^{3}H-AMINO ACIDS IN THE PRESENCE OF AN EXCESS OF tRNA

Age of adults (days)	Initial rate of aminoacylation*
1	192 ± 15
21	318 ± 23
40	372 ± 25

*pC per minute incorporated into tRNA by one mg of cytosol protein during the initial, straight-line phase of aminoacylation, in the presence of 4.5 A_{260} units of tRNA.

TABLE 4. EFFECT OF AGE ON tRNA LEVEL AND ON AMINOACYLATION ABILITY OF tRNA

Age of adults (days)	μg of tRNA/fly*	Total ^{3}H-amino acid bound by one A_{260} unit of tRNA** (pC)
1	4.3	17,540.
21	3.4	14,625.
40	3.2	10,333.

*The tRNA was isolated as described in the Materials and Methods section.

**Cytosol from 1-day-old adults was supplemented with 4.0 A_{260} units of tRNA from adults of the indicated ages, and the total exte: t of aminoacylation was determined with the reaction system described Materials and Methods.

free protein synthesis decreased approximately 70%, there was a decrease of no more than 20% in the rate of aminoacylation of tRNA, although the extent of aminoacylation decreased 36% in 21-day-old adults. Comparison of the rate of aminoacylation in the cytosol with the rate at which amino acids are incorporated into protein (Webster and Webster, 1979) revealed that, under essentially similar conditions, the rate of aminoacylation was four times as rapid as the rate of incorporation of amino acids into protein. Thus, aminoacylation would have to decrease markedly before it would be expected to be a major cause of a decrease in protein synthesis as large as 70%. The only exception would be if the rate of aminoacylation of one or more amino acids would decrease essentially to zero. However, the present findings indicate that this is not the case. Therefore, although a decrease in aminoacylation may be a contributor to the age-related decrease in protein synthesis in *Drosophila*, it may not be the major factor in the decrease. However, if a very small amount of a particular tRNA were present in the cytosol and there was a great demand for that amino acid during translation, decreased aminoacylation of that tRNA could be a major contributor to decreased protein synthesis.

The contribution of decreased aminoacylation to decreased protein synthesis can probably be evaluated better when the effects of age are known on the other three stages of protein synthesis. In unpublished experiments, we have found only a small decrease in initiation of translation in aging *Drosophila*. However, our observation (Webster and Webster, 1979) that the polyuridylate-directed incorporation of phenylalanine into polymer (under conditions where initiation is probably largely bypassed [Nakamoto and Kolakofsky, 1966]), exhibited an age-related decrease comparable with the decrease in protein synthesis, suggests that decreased peptide chain elongation may be an important factor in the decreased protein synthesis. This agrees with the report of Moldave *et al.* (1979) of an age-related decrease in peptide chain elongation in rats.

The age-related decrease in the ability of tRNA to accept amino acids is of considerable interest, however. Is the decreased ability due to an age-related formation of enzymes that alter the tRNA, or is it due to a shut-down of tRNA synthesis? Since the formation of tRNA appears to be a simpler process than translation, involving principally transcription and processing, an age-related stop in tRNA synthesis would provide a convenient system for the study of the control of gene expression during aging.

SUMMARY

Aminoacylation of tRNA was measured in extracts of *Drosophila melanogaster* of in-

Vol. 16 1981

creasing age. During the period when the rate of protein synthesis decreased by 70%, the rate of aminoacylation by a mixture of amino acids decreased no more than 20%. The ability of tRNA to accept amino acids decreased 36%. Aminoacylation of tRNA by each of the 20 amino acids showed age-related decreases in rate of up to 38% and decreases in extent of aminoacylation ranging up to 54%. Aminoacylation enzymes did not exhibit an age-related decrease in activity. However, both the levels of tRNA in the cytosol and the ability of the tRNA to accept amino acids decreased with age, and probably account for the observed decreases in aminoacylation.

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THE INFLUENCE OF AGE ON THE INCREASED ACTIVITY OF 3-HYDROXY-3-METHYLGLUTARYL-CoA REDUCTASE IN REGENERATING LIVERS OF PARTIALLY HEPATECTOMIZED RATS

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INTRODUCTION

Changes in the incidence of arteriosclerosis, the plasma concentration of cholesterol, and the synthesis of cholesterol occur with age. Various groups have demonstrated that the synthesis of cholesterol in the liver of rats decrease with age (Bloch *et al.*, 1946; Yamamoto and Yamamura, 1971; Hotta, 1980; Kritchevsky, 1980). Although our studies showed similar changes with age, our results also indicated that the hepatic synthesis of cholesterol was relatively low in very young rats before increasing to a maximum and then decreasing as the rats became older (Hotta, 1980). It seemed likely that these changes in cholesterol synthesis might be accompanied by corresponding changes in the activities of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme in the biosynthesis of cholesterol. Similarly, increases in HMG-CoA reductase activities were expected in regenerating livers to correspond with the increased synthesis of cholesterol that was observed in regenerating livers of partially hepatectomized rats (Takeuchi *et al.*, 1976). We therefore utilized rats of various ages to determine the possible changes in HMG-CoA reductase activities with age in normal and regenerating livers.

METHODS AND PROCEDURES

Female rats of the Wistar strain were obtained from the Gerontology Research Center (NIA), Baltimore, Maryland. The rats used in these studies were 3 months, 13 months, and 24 months of age. There were 6 rats in each age group. All rats were maintained on a commercial rat chow in a room with a 12-hour light/dark cycle (light from 7 a.m. to 7 p.m.). The rats were partially hepatectomized or killed between 1 p.m. and 3 p.m. The rats were weighed and partially hepatectomized (70%) under ether anesthesia (Higgins and Anderson, 1931). The removed livers were weighed and frozen for later analysis. At 48 hours after surgery, the rats were weighed and killed. The remaining livers were removed, weighed and frozen for later analysis. The livers removed at surgery served as the control livers for the regenerating livers in the same rats.

Portions of the livers were homogenized with a Potter-Elvehjem homogenizer with a Teflon® pestle in three volumes of a buffer containing 50 mM potassium phosphate, pH 7.4, 30 mM EDTA, 250 mM NaCl, and 1 mM dithiothreitol. After centrifuging the homogenate at 12,000 g for 15 min, the supernate was centrifuged at

TABLE 1. LIVER PROTEIN BEFORE AND AFTER PARTIAL HEPATECTOMY

Rat		Liver		Liver protein	
Age(mon)	Body weight(g)	Removed(g)	At sacrifice(g)	Control(mg/g)	After hepatectomy(mg/g)
3	196.7 ± 13.5	4.97 ± 0.67	3.60 ± 0.42	233.2 ± 10.2	211.4 ± 5.2
13	318.8 ± 24.5	6.52 ± 0.61	4.61 ± 0.42	220.8 ± 1.6	214.2 ± 1.0
24	392.7 ± 58.9	7.61 ± 0.80	5.52 ± 0.91	212.4 ± 1.4	205.4 ± 3.7

Six rats in each age group were partially hepatectomized. Liver tissue removed at surgery and that remaining 48 hours later were analyzed for protein content. Results are averages ± S.E.M.

105,000 g for 45 min at 4°C. The pelleted microsomes were suspended in fresh buffer and made up to one half the volume of supernate from which they were sedimented. Portions of the microsomal preparations were assayed for HMG-CoA reductase activity according to the method described by Shapiro *et al.* (1974). Microsomes in 100 μl of the homogenizing buffer were preincubated for 5 min at 37°C. The activity of HMG-CoA reductase was then assessed by adding 50 μl of a solution containing 450 nmoles of NADP, 4.5 μmoles of glucose-6-phosphate, 0.3 I.U. of glucose-6-phosphate dehydrogenase, and 50 nmoles of DL-[3-^{14}C]-HMG-CoA (specific activity 880 dpm/nmole). Total incubation volume was 150 μl. At 15 min, 50 μl 6 N HCl and 10 μl [5-^{3}H]-mevalonolactone (22,000 dpm) were added and the mixture was incubated for 30 min at 37°C. The mevalonolactone was extracted with toluene or separated by thin layer chromatography as described (Phillip and Shapiro, 1979). The ^{14}C incorporated into mevalonate was counted with a Beckman LS-7000 scintillation counter. The activity of HMG-CoA reductase was calculated from this conversion after correction for the recovery of mevalonolactone, and expressed as the pmoles mevalonate formed per min per mg protein. Portions of the livers were analyzed for proteins, cholesterol, triglyceride and total glyceride contents. Proteins were measured by the method of Lowry (1951). Cholesterol was extracted with hexane from an alcoholic-KOH saponified aliquot and measured by the colorimetric method described by Searcy (1960). Triglycerides and phosphoglycerides were extracted with acetone-ethyl acetate (Slayback, Cheung, and Geyer, 1977). An aliquot of this extract was used to measure the total glycerides after KOH-hydrolysis and colorimetric determination of the released glycerol as described by Fletcher (1968). A second aliquot was used to extract the triglycerides with nonane as described by Royer and Ko (1969). The triglycerides were then quantitated (Fletcher, 1968) and this amount was subtracted from the total glycerides. The difference represented the phosphoglycerides. All the glyceride values are expressed as milligram equivalents of triolein, since triolein was used as a standard in these procedures.

NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, dithiothreitol, and HMG-CoA were obtained from the Sigma Chemical Co. (St. Louis). DL-[3-^{14}C]-HMG-CoA and [5-^{3}H]-mevalonolactone were obtained from the New England Nuclear Corp. (Boston).

RESULTS

With increasing age, the rats gained weight and had larger livers (Table 1). The increase in liver size paralleled the increase in body weight such that the proportion of liver to body weight remained relatively constant. Similarly, the protein content (about 200 mg per g liver) was essentially the same in the livers of the different age groups and, furthermore, did not change significantly in the regenerating livers.

Analysis of the lipids in these livers showed that the concentration of cholesterol increased with age of the rat and the differences were maintained in the regenerating livers (Table 2). Although the phosphoglyceride concentrations did not vary with age, they increased markedly (about 2.5 fold) in the regenerating livers. Similarly, the triglyceride concentrations increased greatly in the regenerating livers and, additionally, showed a tendency to be greater in the livers of older rats; the triglyceride accumulations were evident grossly by the noticeable fatty appearance of the regenerating livers.

Assay of HMG-CoA reductase showed that before hepatectomy, the livers of the

Vol. 16 1981

TABLE 2. LIVER LIPIDS BEFORE AND AFTER PARTIAL HEPATECTOMY

Age (mon)	Cholesterol		Phosphoglycerides		Triglycerides	
	Control	After hepatectomy	Control	After hepatectomy	Control	After hepatectomy
3	1.318 ± 0.041	1.590 ± 0.123	12.25 ± 0.55	30.24 ± 3.45	3.66 ± 1.18	67.23 ± 14.51
13	1.846 ± 0.106	1.855 ± 0.102	10.85 ± 0.75	26.00 ± 2.99	3.42 ± 0.26	94.77 ± 3.35
24	2.007 ± 0.109	2.017 ± 0.118	11.24 ± 0.62	29.94 ± 1.05	5.57 ± 1.18	102.95 ± 20.18

Liver tissue removed at surgery and that remaining 48 hours after partial hepatectomy were analyzed for their lipid contents. Results are expressed as average mg lipid/g liver ± S.E.M.

13-and 24-month-old rats had about the same activity while the activity in the 3-month-old rats tended to be lower. All of the regenerating livers showed significant increases in HMG-CoA reductase activity. This increase was the greatest in the 13-month-old rats (79.5%) while the least increase occurred in the regenerating livers of the 24-month-old rats (28.7%). The regenerating livers in the 3-month-old rats showed an increase in HMG-CoA reductase of 49.8%.

DISCUSSION

The results show that the older rats weighed more, had bigger livers, and had higher hepatic cholesterol concentrations. These findings were similar to our previously reported findings (Hotta, 1980). The increases in liver weights were proportional to the increases in body weights. The increases in cholesterol concentration indicated that there were accumulations of cholesterol in the livers of older rats. This was in contrast to the relatively constant concentrations of proteins and total glycerides in the livers of rats of various ages.

The regenerating liver appeared to accumulate phosphoglycerides and triglycerides, but showed no change in cholesterol concentration. After hepatectomy, the phosphoglyceride concentration increased from 10–12 mg/g liver to about 26–30 mg/g liver, while the triglyceride concentration increased from about 3–5 mg/g liver to 67 mg/g in 3-month-old rats, 95 mg/g in 13-month-old rats and 103 mg/g in 24-month-old rats. These findings were compatible with the observations of Murray *et al.* (1980), that the average size of the hepatocytes increased in regenerating livers; that is, the accumulation of phosphoglycerides and triglycerides probably contributed to the enlargement of the hepatocytes.

Our earlier studies showed that the hepatic synthesis of cholesterol was low in the very young rats, increased to a maximum, and then decreased as rats aged (Hotta, 1980). In contrast, the rate-limiting HMG-CoA reductase activities in the control livers of the rats in the present studies did not show any significant differences in enzyme activities from one age group to another. That the HMG-CoA reductase activity only tended to be lower in the 3 month old rats seemed understandable because these rats were older than those used in the previous studies. It is possible that, if younger rats were used, we may have found lower enzyme activities to correspond to the lower cholesterol synthesis we observed in livers of very young rats. However, the unchanged activity of HMG-CoA reduc-

TABLE 3. EFFECT OF PARTIAL HEPATECTOMY ON MICROSOMAL HMG-CoA REDUCTASE ACTIVITY

Rat age(mon)	Before hepatectomy	After hepatectomy	Percent increase in regenerating liver
3	1607 ± 141	2160 ± 277	49.8[a] ± 20.1
13	1873 ± 163	3244 ± 376	79.5[b] ± 22.3
24	1892 ± 95	2256 ± 127	28.7[c] ± 6.3

Microsomes, isolated from livers removed at surgery and from livers remaining 48 hours after partial hepatectomy, were assayed for HMG-CoA reductase activity. The results are average pmoles mevalonate formed/min/mg protein ± S.E.M.

[a] $p < 0.05$
[b] $p < 0.02$
[c] $p < 0.01$

tase in the livers of the 24-month-old rats was not expected in view of the decreased rates of cholesterol synthesis previously observed in old rats (Bloch *et al.*, 1946; Yamamoto and Yamamura, 1971; Hotta, 1980; Kritchevsky, 1980). This apparent non-correlation of enzyme activity to cholesterol synthesis suggest that the decrease in cholesterol synthesis in older rats may involve other factors in addition to HMG-CoA reductase.

Partial hepatectomy led to significant increases in the HMG-CoA reductase activities in the regenerating livers in all age groups. The greatest increase (79.5%) occurred in the 13-month-old rats, with a smaller increase (49.5%) in the 3-month-old rats, and the smallest increase (28.7%) in the 24-month-old rats. It thus appeared that the abilities of the regenerating livers to increase their HMG-CoA reductase level depended upon the age of the rats. It was further noted that the different increases in hepatic enzyme levels in rats of different ages seemed to correlate with the amount of mitotic activity evident in the regenerating livers (unpublished observations); that is, the livers showing the highest increases in HMG-CoA reductase activities showed the highest mitotic activities. This suggested that the increased activity of HMG-CoA reductase in regenerating livers was closely associated with the mitotic activity of the cell.

Our findings that the regenerating liver had increased amounts of phosphoglycerides and triglycerides in addition to an increase in HMG-CoA reductase activity are consistent with the suggestions that increases in lipid metabolism were necessary for the growth and development of rapidly proliferating cells (Kandutsch and Chen, 1977; Resch and Ferber, 1973). The increased HMG-CoA reductase activity correlated with the observed increase in incorporation of acetate into cholesterol in regenerating livers (Takeuchi *et al.*, 1976), while an increased synthesis of phosphoglycerides probably accounted for their accumulation in the regenerating livers. Whether there was an increase in the *de novo* synthesis of triglyceride or was an increase in the reassembly of fatty acids mobilized from the adipose tissues to the liver or were increases in both remains unknown.

Vol. 16 1981

SUMMARY

The activity of 3-hydroxy-3-methylglutaryl-CoA reductase and the lipid composition were determined in normal and regenerating livers of rats of various ages. The reductase activities did not differ greatly among the livers of the 3-, 13-, and 24-month old rats. However, the reductase activities increased significantly in the regenerating livers of these rats following partial hepatectomy. The percent increase in enzyme activity varied with the age of the rat. The greatest increase (79.5%) occurred in the livers of the 13-month-old rats, with lesser increases occurring in the 3-month-old rats (49.5%) and in the 24-month-old rats (28.7%). Phosphoglyceride and triglyceride concentrations were also increased in the regenerating livers. The results suggested that lipid metabolism was stimulated in the regenerating liver after partial hepatectomy and that the degree of stimulation varied with the age of the rat.

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Vol.
16
1981

END OF
VOL.